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Studies on Cecum Digestion

Part IV. On the Movement of Cecal Contents in the Rabbit (1)

By Ichiro YOSHIHARA* and Makoto KANDATSU**

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Received January 18, 1960

For the study of cecum digestion, the movement of cecal contents in the rabbit with abdominal window was studied by injecting ^{35}S-barium sulfate into the cecum. The fecal output and the distribution of radioactivity in cecum, indicated that the hard and soft feces in the rabbit are formed from the same cecal contents. A new assumption was thus suggested to explain the formation of the hard and soft feces.

INTRODUCTION

Although there are only few reports concerning the problem of how cecal contents enter and get out, how long they remain in the cecum, information in regard of these facts is of fundamental importance for clarification of the function of cecum.

It has been reported that the rabbit excretes hard and soft feces almost completely consuming the soft type of which the composition is comparable to that of the cecal contents$^{1\sim5)}$. The soft feces and the cecal contents have close relation with each other, and it is on this point that Eden$^{2)}$ and Thacker et al.$^{6)}$ have postulated i.e., the soft feces would be formed from the contents in the deeper regions of the cecum and the hard feces would be formed from the contents by-passed the cecum.

In this report data on the movement of cecal contents and the origin of the soft feces are presented. These facts can't be explained by the former assumption and therefore a new assumption is suggested here.

EXPERIMENTAL AND RESULTS

Four Japanese White Rabbits with abdominal window were used. The rabbits were completely restored in about a week after a surgical operation of sewing the abdominal window. For prevention of coprophagy the rabbit was provided with a large wooden collar (27.5 cm in diameter) in Expt. I, and in Expts. II, III and IV the rabbits were stanchioned. All rabbits were fed on 120～150 g of a diet, consisting of an equal proportion (by weight) of wheat bran, a commercial mixed feed* for dairy cows and hay-meal, every morning and maintained their initial body weights.

When the rabbits were completely restored, ^{35}S-barium sulfate suspended in dilute agar solution (Expts. I and II) or in thin starch paste (Expts. III and IV), was injected into the cecum with an injection-syringe. After successful injections the rabbits took their diet at once and the feces were excreted as usual. The feces were collected whenever they were properly stored in the inverted conical nylon net suspended under the cage. Each fecals ample was dried at 70～80°C and then ashed in an evaporating dish to determine the ash content and 100 mg of thoroughly mixed ash was taken in a dish for assay (25×7 mm). After the addition of a few drops of distilled water, the pasty ash was mashed with a slender glass rod and a small amount of distilled

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1) H. Madsen, *Nature*, **143**, 981 (1939).
2) A. Eden, *Nature*, **145**, 36, 628 (1940).
3) I. Frank, U. Hadeler and W. Harder, *Pflügers Arch.*, **253**, 173 (1951).
4) M. Kandatsu, I. Yoshihara and T. Yoshida, *Japan. J. Zootech. Sci.*, **29**, 365 (1959).
5) T. Yoshida and M. Kandatsu, *ibid.*, **30**, 151 (1959).
6) E.J. Thacker and C.S. Brandt, *J. Nutrition*, **55**, 375 (1955).

* This mixed feed contained wheat bran, soybean oil meal, linseed oil meal, coconut oil meal, potato starch pulp, citrus pulp, condensed fish solubles, calcium carbonate and sodium chloride. The composition of this mixed feed was as follows: moisture 11.4, crude protein 18.4, crude fat 4.0, N-free extract 48.4, crude fiber 9.1 and ash 8.7 per cent.

water was added to the dish washing a rod. The dish was evaporated to dryness under an infra-red lamp. Mashing and drying were repeated three times[7] and radioactivity was assayed with a Radiation Counter (Aloka, Model DC-3).

Experiment I

Rabbit O (female, 39 months of age) was operated on October 18, 1955. ^{35}S-barium sulfate was successfully injected three times repeatedly: for the first time on November 1, the second time on November 5 and the third time on November 9. The results are shown in Tables I-1, I-2 and I-3. Rabbit O was slaughtered seven hours after the third injection and the cecal contents were divided into 6 parts as shown in Table I-4. The results are shown in Table I-4.

Experiment II

Rabbit P (female, 7 months of age) was operated on October 22, 1956. ^{35}S-barium sulfate was successfully injected only once on November 5. The results are shown in Table II.

Experiment III

Rabbit Q (female, 12 months of age) was operated on May 9, 1958. ^{35}S-barium sulfate was injected at 5.15 p.m. on May 13, but on account of failure in injection, peristalsis stopped till about 10.00 p.m. The results are shown in Table III-1. On May 19 the rabbit was slaughtered forty minutes after injection and after taking out the cecum, the cecal contents were frozen and divided into 6 parts (Table III-2). In this experiment the amount of ash in contents of the appendix was so small that the radioactive assay was carried out with 37 mg of ash. The results are shown in Table III-2.

Experiment IV

Rabbit R (female, 12 months of age) was operated on May 28, 1958. ^{35}S-barium sulfate was injected at 4.50 p.m. on June 2. However on account of failure in injection, peristalsis stopped till 6.40 p.m. The rabbit was slaughtered forty minutes after resumption of peristalsis and after taking out the cecum, the frozen cecal contents were divided into 10 parts (Table IV). The results are shown in Table IV.

DISCUSSION

The blank test showed 1 and 3 C.P.M. per 100 mg of ash of the hard and soft feces and it may therefore be recognized that the radioactivity of fecal ash is practically due to mixed ^{35}S-barium sulfate. For the purpose of diminishing differences of self-absorption among dishes,

TABLE I-1. FECAL EXCRETION OF ACTIVITY OF ^{35}S-BARIUM SULFATE INJECTED INTO THE CECUM

Injected portion, 14th segment
Injected at 0.30 p.m.
Amount injected, 1.1891g

Number of sample	Date of collection		Kind of feces	Amount of ash	C.P.M. per 100mg of ash
1	Nov. 1	3.30 p.m.	hard	417.7mg	65
2		5.00 p.m.	hard	594.8	171
3		6.40 p.m.	hard	553.4	116
4		7.40 p.m.	hard	525.7	94
5		8.50 p.m.	hard	697.8	74
6		9.30 p.m.	hard	574.8	55
7		10.40 p.m.	soft	408.0	39
8	Nov. 2	2.00 a.m.	soft	152.0	28
9		9.00 a.m.	soft	590.2	23
10		9.00 a.m.	hard	1052.6	18
11		10.30 a.m.	hard	500.1	20
12		11.30 a.m.	hard	580.9	23
13		1.40 p.m.	hard	567.1	25
14		3.15 p.m.	hard	677.2	18
15		7.00 p.m.	hard	479.1	14
16		8.00 p.m.	hard	416.4	13
17*	Nov. 3	5.05 a.m.	hard	278.3	9
18		5.05 a.m.	soft	431.9	8
19		7.40 a.m.	hard	598.8	6
20		10.00 a.m.	hard	396.4	4

* Hard feces were under soft feces (No. 18) in the net.

TABLE I-2. FECAL EXCRETION OF ACTIVITY OF ^{35}S-BARIUM SULFATE INJECTED INTO THE CECUM

Injected portion, 12th or 13th segment
Injected at 0.00 p.m.
Amount injected, 1.1704g

Number of sample	Date of collection		Kind of feces	Amount of ash	C.P.M. per 100mg of ash
1	Nov. 5	1.30 p.m.	hard	522.0mg	96
2		3.40 p.m.	hard	267.7	118
3		3.40 p.m.	soft	420.5	126
4		4.30 p.m.	hard	527.3	105
5		5.40 p.m.	hard	548.4	88
6		6.40 p.m.	hard	201.7	62
7		6.40 p.m.	soft	257.1	52
8		8.50 p.m.	soft	362.2	55
9		10.20 p.m.	soft	343.1	50
10	Nov. 6	3.15 a.m.	hard	1232.9	47
11		3.15 a.m.	soft	549.0	29
12		5.45 a.m.	soft	404.5	27
13		5.45 a.m.	hard	367.8	33
14		9.10 a.m.	hard	1566.0	34
15		1.30 p.m.	hard	582.0	23
16		3.40 p.m.	hard	582.5	17
17		7.20 p.m.	hard	423.8	14
18		7.50 p.m.	hard	484.3	12
19	Nov. 7	6.30 a.m.	hard	295.7	8
20		6.30 a.m.	soft	467.7	7
21		8.40 a.m.	hard	499.4	5
22		10.00 a.m.	hard	749.9	2

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[7] M. Kandatsu and I. Yoshihara, *J. Japan. Biochem. Soc.*, **27**, 541 (1955).

radioactive assay was carried out with a constant amount of fecal ash, but the following facts may be noteworthy for discussion of our results obtained. Generally, the ash of hard feces absorbs ^{35}S-β- particle more effectively than that of soft feces and the degree of self-absorption becomes higher with the increase of ^{35}S-barium sulfate content in fecal ash[8].

The results obtained can be divided into 2 groups: the one is related to the excretion of radioactivity injected into cecum (Table I-1, I-2, I-3, II and III-1) and the other to the distribution of radioactivity in the cecum (Tables I-4, III-2 and IV). The results concerning the former show a general tendency that C.P.M. of fecal ash increases and decreases gradually showing a peak regardless of 2 types of feces. This tendency was not disturbed by the abnormal excretion of hard and soft feces (Table I-2 No. 10 and Table I-2 No. 3) and a peak appeared about 4~6 hours after successful injection into the middle regions of the cecum (Table I-1 No. 2, I-2 No. 3 and II No. 3).

TABLE I-3. FECAL EXCRETION OF ACTIVITY OF ^{35}S-BARIUM SULFATE INJECTED INTO THE CECUM

Injected portion, 2nd segment
Injected at 11.45 a.m.
Amount injected, 1.1586g

Number of sample	Date of collection		King of feces	Amount of ash	C.P.M. per 100mg of ash
1	Nov. 9	1.40 p.m.	hard	206.0mg	2
2		2.30 p.m.	hard	581.5	202
3		3.15 p.m.	hard	295.1	139
4		3.35 p.m.	hard	260.7	105
5		4.15 p.m.	hard	340.9	90
6		4.45 p.m.	hard	284.6	81
7		5.45 p.m.	hard	290.0	68
8		6.10 p.m.	hard	282.8	63
9		6.45 p.m.	hard	198.8	55

TABLE I-4. DISTRIBUTION OF ACTIVITY IN CECAL CONTENTS OF THE RABBIT SLAUGHTERED 7 HOURS AFTER INJECTION OF ^{35}S-BARIUM SULFATE INTO THE 2ND SEGMENT OF CECUM

Portion of cecal contents	Amount of ash	C.P.M. per 100mg of ash
1st~4th segment	708.3mg	44
5th~8th seg.	668.6	38
9th~12th seg.	653.3	39
13th~16th seg.	572.4	55
17th~20th seg.	517.6	41
21st seg.~appendix	200.7	43

TABLE II. FECAL EXCRETION OF ACTIVITY OF ^{35}S-BARIUM SULFATE INJECTED INTO THE CECUM

Injected portion, 9th or 10th segment
Injected at 10.40 a.m.
Amount injected, 0.4928g

Number of sample	Date of collection		Kind of feces	Amount of ash	C.P.M. per 100mg of ash
1	Nov. 5	0.30 p.m.	hard	536.1mg	3
2		3.45 p.m.	hard	424.7	30
3		4.30 p.m.	hard	426.6	46
4		5.30 p.m.	hard	591.5	41
5		6.30 p.m.	hard	576.1	33
6		7.20 p.m.	hard	528.4	26
7		8.05 p.m.	hard	482.1	16
8		9.20 p.m.	hard	580.4	15
9		10.40 p.m.	hard	776.5	13
10	Nov. 6	10.30 a.m.	soft	1856.7	4
11		10.30 a.m.	hard	1193.0	4
12		0.05 p.m.	hard	472.1	3
13		2.15 p.m.	hard	599.4	2

TABLE III-1. FECAL EXCRETION OF ACTIVITY OF ^{35}S-BARIUM SULFATE INJECTED INTO THE CECUM

Injected portion, 12th segment
Injected at 5.15 p.m.
Amount used, 1.1132g

Number of sample	Date of collection		Kind of feces	Amount of ash	C.P.M. per 100mg of ash
1	May 13	12.00 p.m.	hard	1087.9mg	38
2	May 14	8.30 a.m.	hard	1032.7	42
3		8.30 a.m.	soft	1150.1	28
4		11.20 a.m.	hard	616.8	26
5		3.15 p.m.	hard	580.8	22
6		8.45 p.m.	hard	649.4	14
7	May 15	10.00 a.m.	hard	1179.7	6
8		10.00 a.m.	soft	1481.6	4
9		2.00 p.m.	hard	708.6	4

TABLE III-2. DISTRIBUTION OF ACTIVITY IN CECAL CONTENTS OF THE RABBIT SLAUGHTERED 40 MINUTES AFTER INJECTION OF ^{35}S-BARIUM SULFATE INTO THE 12TH SEGMENT OF CECUM

Amount used, 0.4055g

Number of sample	Portion of cecal contents		Amount of ash	C.P.M. per 37mg of ash
1	1st ~ 9th segment,	Interior*	467.0mg	58
2	1st ~ 9th seg.,	Exterior*	774.6	59
3	10th~15th seg.,	All	1217.4	61
4	16th~25th seg.,	M-side*	238.3	65
5	16th~25th seg.,	O-side*	298.1	63
6	Appendix	All	39.5	48

* Show in Fig. 1,

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[8] I. Yoshihara, A. Sugisaki and M. Kandatsu, *J. Agr. Chem. Soc. Japan*, **34**, 563 (1860).

In spite of considering the facts mentioned above, this tendency remains unchanged. The results concerning the latter show an approximately uniform distribution of radioactivity in the cecum. These two general tendencies can't be explained by the former assumption, but indicate the fact that the hard and soft feces are formed from identical cecal contents.

TABLE IV. DISTRIBUTION OF ACTIVITY IN CECAL CONTENTS OF THE RABBIT SLAUGHTERED 40 MINUTES AFTER INJECTION OF ^{35}S-BARIUM SULFATE INTO THE 15TH OR 16TH SEGMENT OF CECUM

Amount used, 0.5229g

Number of sample	Portion of cecal contents	Amount of ash	C.P.M. per 40mg of ash
1	1st~3rd segment, All	221.8mg	50
2	4th~11th segment, M-side, Interior*	239.0	65
3	4th~11th segment, M-side, Exterior*	379.2	49
4	4th~11th segment, O-side, Interior*	281.2	49
5	4th~11th segment, O-side, Exterior*	529.7	55
6	12th~14th segment, Internal organ side*	284.6	54
7	12th~14th segment, Abdominal window side*	294.6	51
8	15th~17th segment, All	370.5	58
9	18th~23rd segment, All	419.3	65
10	24th seg.~appendix, All	42.9	48

* Shown in Fig. 1,

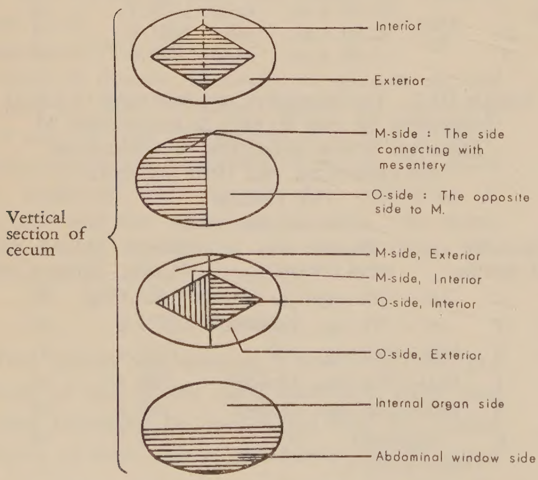

FIG. 1. Sampling Methods of Frozen Cecal Contents.

In accordance with the former assumption, the differences between the composition of the hard and soft feces must arise in the cecum. Thacker and Brandt[6] suggested a protein containing cecal secretion to explain the compositional differences in the hard and soft feces. In accordance with the fact mentioned above, a possible physiological mechanism must be assumed to explain the formation of the hard and soft feces. The remarkable absorption in the large intestine of the rabbit has been shown in a few reports[9,10] and we believe that the absorption in the large intestine will be an essential factor of clarifying the mechanism. It may be probable that the regular slow passage of the cecal contents is responsible for the formation of the hard feces and a more rapid discharge is responsible for that of the soft feces. In Expt. III, the amount of ^{35}S-barium sulfate was determined[8]. In spite of higher activity of No. 3 (soft feces) than that of No. 4 (hard feces), the amount of ^{35}S-barium sulfate was lesser in No. 3. In Table I-2, No. 11 and No. 12 (soft feces) showed lower activity than No. 13 and No. 14 (hard feces). These results show the possibility that the hard feces are formed because they are more absorbed than the soft feces. Thus it may be possible that the composition of the less absorbed soft feces is comparable to that of the cecal contents[1~5].

Yasukawa and Nagano[11] reported that the contents from the small intestine proceed spirally towards Apex caeci along the cecal wall and turn back towards Basis caeci passing through the central portion. Though their result directed

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9) T.C. Huang, H.E. Ulrich and C.M. McCay *J. Nutrition*, **54**, 621 (1954).

10) I. Yoshihara and A. Sugisaki, *Japan. J. Zootech. Sci.*, **31**, 9 (1960).

11) M. Yasukawa and K. Nagano, *Bull. Faculty of Agr., Kagoshima Univ.*, No. 6, 157 (1957).

our attention to the sampling method of the cecal contents, the results obtained by us (Tables III-2 and IV) did not support their result, showing an approximately uniform distribution of radioactivity regardless of sampling methods. These results show a very effective mixing and and movement of the entire cecal contents.

Acknowledgements The authors wish to express their hearty thanks to Ass. Prof. M. Ikeda and Mr. T. Hayama, Univ. of Tokyo, for their kind operation of sewing the abdominal window in Rabbit I and to Mr. K. Miyano, Univ. of Niigata, for his kind guidance in the surgical operation of sewing the abdominal window.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 547~554, 1960]

Determination of Nucleic Acids in Yeast from the Viewpoint of Elution Behavior of Acid-Soluble Compounds*

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Received January 19, 1960

It was reported[1)] previously that Ogur and Rosen's method for the determination of nucleic acids should be applied to fresh or durable yeast, after a freezing pretreatment with dry ice and ether. The fractionation of nucleic acids from the frozen yeast should precede with a complete elimination of alcohol-ether-soluble and acid-soluble compounds. It has been found that in Ogur and Rosen's method, the elimination of the former compounds is complete, but the removal of acid-soluble compounds is unsatisfactory in the case of yeast.

In order to improve this defect, the best conditions for the elution of acid-soluble compounds have been investigated in detail, and it has been found that water is the most suitable solvent.

Many difficulties have been encountered in applying the method for the determination of nucleic acids in animal tissues to fresh microorganisms. One of the authors applied Ogur and Rosen's method[2)] to fresh or durable yeast and found that this method was unsatisfactory, because RNA could not be extracted completely by their procedure. Therefore, this method was modified by introducing the freezing pretreatment with dry ice and ethyl ether, as reported in the previous paper[1)].

Among the procedures involved in their method, the removal of alcohol-ether-soluble and acid-soluble compounds is very important, which precedes the fractionation of nucleic acids, because alcohol-ether-soluble and acid-soluble compounds absorb ultraviolet light at almost the same wave-lengths as RNA does.

The conditions for the complete removal of such compounds have been investigated, and it has been found that the use of water, in

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The following abbreviations are used: P_o, orthophosphoric acid phosphorus; P_p, polyphosphoric acid phosphorus; P_t, total phosphorus; RNA, ribonucleic acid; RNAP, ribonucleic acid phosphorus; DNA, disoxyribonucleic acids; DNAP, disoxyribonucleic acid phosphorus; PCA, perchloric acid; TCA, trichloroacetic acid.

* The content of this report was previously announced in the annual meeting of the *Agr. Chem. Soc. Japan* held on April 3rd, 1958.

1) Y. Kuroiwa and Y. Horie, This Bulletin, **19**, 35 (1955).

2) M. Ogur and G. Rosen, *Arch. Biochem. Biophys.*, **25**, 262 (1950).

place of 0.2 N perchloric acid, is most suitable for the elution of acid-soluble compounds. By the use of both the freezing pretreatment and the extraction with water, a modified method for the determination of nucleic acids in fresh or durable yeast is presented in this papar.

Method for the Determination of Nucleic Acids in Fresh or Durable Yeast.

Two grams of pressed yeast, equivalent to about 500 mg of dry matter, is weighed in a centrifugal tube, the water content adjusted to 80% and 2 g of dry ice and 2 ml of ethyl ether are added, and the whole is frozen at −70°C overnight in a Dewer vessel filled with dry ice[1].

After the frozen yeast is melted in 20 ml of ice-cold ethanol, the extraction of alcohol-soluble compounds as well as the dehydration of the yeast is repeated three times with 20 ml portions of ice-cold ethanol as rapidly as possible, while the enzyme action is suppressed. Then, the residual yeast is extracted three times with 20 ml portions of ethanol-ether mixture (3: 1 v/v) at 90°C for three minutes to remove alcohol-ether-soluble compounds and to destroy the enzymes involved in the yeast and dried in a centrifugal tube in a desiccater.

This dried sample is extracted for ten minutes with 5 ml of ice-cold water and centrifuged for ten minutes. This process is repeated ten times in order to eliminate acid-soluble compounds completely.

In order to extract RNA, the residue is suspended in 5 ml of 1 N PCA and stored at about 0°C overnight and centrifuged. Further the extraction with 5 ml of 1 N PCA for thirty minutes and the centrifugation for ten minutes are repeated fourteen times. The centrifugates are combined and filled up to 100 ml.

In order to extract DNA, the final residue is suspended in 5 ml of 0.5 N PCA and extracted at 70°C for twenty minutes. This extraction at 70°C is repeated three times and all the centrifugates are combined and made up to 25 ml.

The optical density of RNA is measured spectrophotometrically at 260 mμ with 10 mm absorption cells, after 1 ml of the extract is diluted to 25 ml.

DNA is determined colorimetrically by the use of the reaction with diphenylamine[3] as follows; one volume of the extract is mixed with two volumes of diphenylamine reagent and the mixture is boiled for five minutes and rapidly cooled. The intensity of color is measured spectrophotometrically at 600 mμ after the mixture is allowed to stand for twenty minutes.

When the optical densities for RNA and DNA are expressed as x and y respectively, RNAP and DNAP in 1 g of solid yeast are calculated by the following formulae;

$$\mathrm{RNAP} = 2500 \times x \times 1/0.343 \times w\ \mu\mathrm{g}.$$

$$\mathrm{DNAP} = 75 \times y \times 1/0.047 \times w\ \mu\mathrm{g}.$$

where, w indicates the weight of the sample on dry basis in grams, and 0.343 or 0.047 indicates the optical density for 1 μg RNAP or 1 μg DNAP per ml.

EXPERIMENTAL

All assay data were expressed per 2 g of pressed yeast throughout this paper. Yeast samples used in these experiments were prepared as follows; 2 g of pressed yeast, well washed with cold water, was weighed and frozen as described in the previous paper[1].

RNA was estimated spectrophotometrically by measureing the optical density at 260mμ and the colorimetric method by orcinol reaction[4] was also used in some cases. DNA was estimated by the colorimetric method with diphenylamine and by the spectrophotometric method at 268 mμ. Phosphorus was estimated by Allen's method[5].

D_{260} indicates the optical density measured spectrophotometrically at 260 mμ using 10 mm absorption cells, when all the extracts are combined and made up to a definite volume.

1) Extraction of Alcohol-Ether-Soluble Compounds.

In order to remove alcohol-ether-soluble compounds, which interfere with the determination of nucleic acids, Ogur and Rosen's method prescribes that the homogenized sample should be extracted twice with ethanol at 4°C, followed by repeated extraction with ethanol-ether mixture (3 : 1 v/v) for three minutes in a boiling water bath.

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[3] Z. Dische, *Microchemie*, **2**, 26 (1930).

[4] W. Mejbaum, *Z. Physiol. Chem.*, **258**, 117 (1930).

[5] R.J.L. Allen, *Biochem. J.*, **34**, 858 (1940).

However, it does not prescribe any relationship between the amount of the sample and the volume of the solvents. For the purpose of working out at the best condition to elute alcohol-ether-soluble compounds, the frozen yeast was extracted under the conditions described in Fig. 1.

It is recognized that alcohol- and alcohol-ether-soluble compounds can be eluted almost completely when extracted each four times.

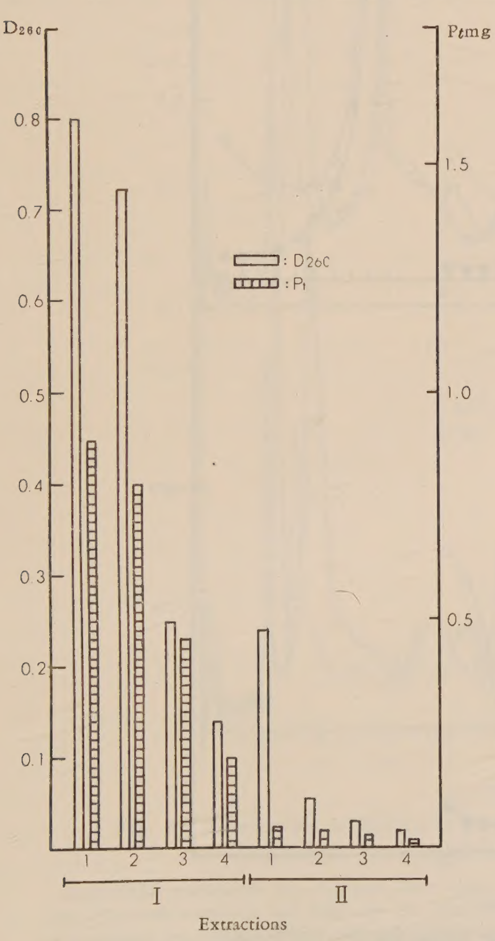

FIG. 1. Aspect of Extraction of Alcohol-Ether-Soluble Compounds.

The frozen yeast was extracted with four 20 ml portions of ethanol at 0°C for 10 min. (I) and the residual yeast was extracted four times each with 20 ml of ethanol-ether mixture (3 : 1 v/v) at 90°C for 3 min. (II). The centrifugate of each run was made up to 100 ml and the optical density and P_t were determined.

Next, the frozen yeast was divided into two parts. One part was extracted three times each with ethanol and with ethanol-ether mixture, and the other part, four times each. After these repeated procedures for the removal of alcohol-ether-soluble compounds, RNAP and P_t in the RNA fraction were estimated. A good agreement between the both values was seen for the two runs.

Hence, the removal of alcohol-ether-soluble compounds is complete, when 2 g of pressed yeast kept frozen overnight is extracted three times with 20 ml portions of ethanol at 0°C for ten minutes and then extracted three times with 20 ml portions of ethanol-ether mixture (3 : 1 v/v) at 90°C for three minutes.

2) Extraction of Acid-Soluble Compounds.

The complete extraction of acid-soluble compounds is an essential procedure in the determination of nucleic acids, since acid-soluble compounds have the ultraviolet light absorption at almost the same wavelengths as RNA does and easily contaminate the RNA fraction. Nevertheless only insufficient information is available concerning the removal of acid-soluble compounds. The procedures ever proposed are various[6~8]; for example, Ogur and Rosen proposed twice extraction with 0.2 N

TABLE I. EXTRACTION OF ACID-SOLUBLE COMPOUNDS WITH PCA, TCA AND WATER.

The yeast sample from which alcohol-ether-soluble compounds were previously removed, was extracted ten times with 5 ml of 0.2 N PCA, 10% TCA or water, at 0°C or 10°C. All the centrifugates of each run were united and the values of D_{260}, P_t and P_p estimated. RNA and DNA were fractionated from the residues of each run obtained above and the quantities estimated.

Extraction of acid-soluble compounds		Acid-soluble fraction			RNA fraction	
with	at	D_{260}*	P_t	P_p	P_t	RNAP
		mg	mg	mg	mg	mg
Water	0°C	0.073(0.22)**	3.82	2.02	3.03	3.03
0.2N PCA	0°C	0.088(0.25)	1.79	0.71	5.04	3.00
10% TCA	0°C	0.091(0.26)	3.50	2.04	3.32	2.99
Water	10°C	0.075(0.22)	3.84	2.10	3.03	3.03
0.2N PCA	10°C	0.126(0.36)	1.98	—	4.93	2.89

* when made up to 1 l.

** each optical density of acid-soluble fraction is calculated temporarily as RNAP.

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[6] W.C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).

[7] L.H. Schmidt and S.J. Thanhauser, *J. Biol. Chem.*, **161**, 83 (1945).

[8] F.J. DiCarlo and A.S. Schultz, *Arch. Biochem. Biophys.*, **17**, 293 (1948).

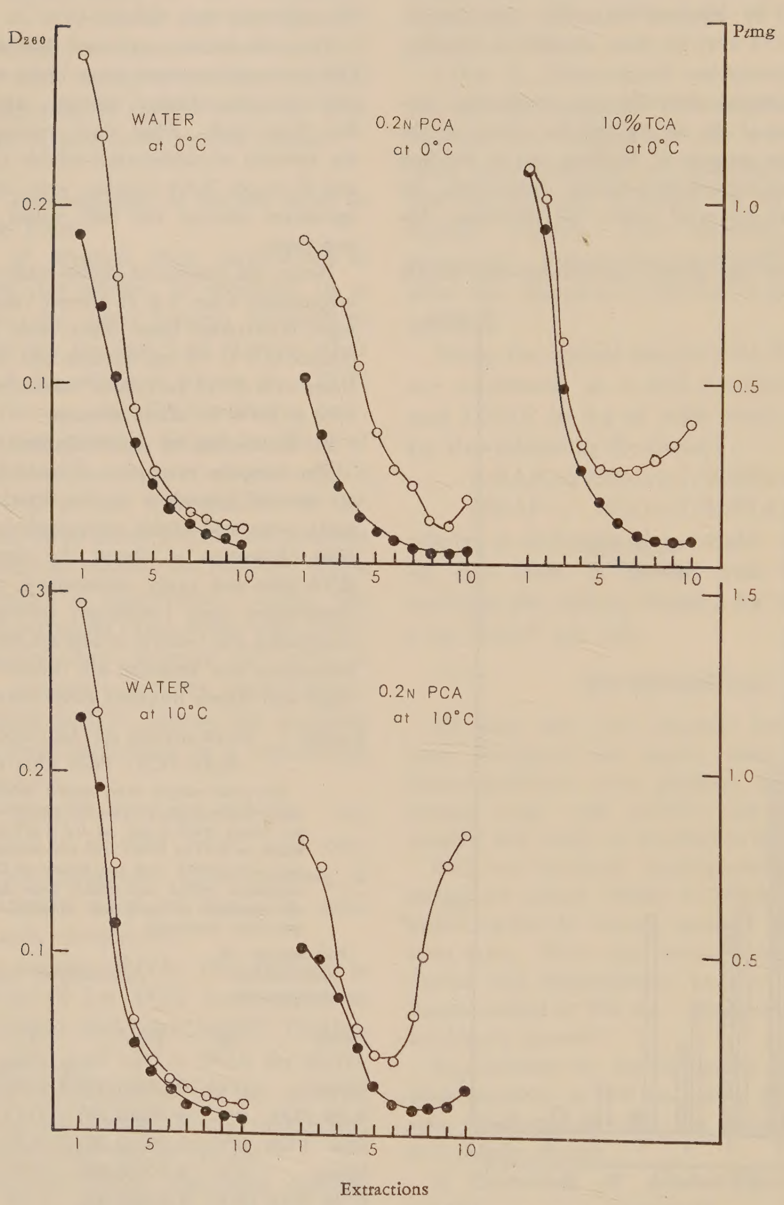

FIG. 2. Further Examination of the Extraction of Acid-Soluble Compounds with TCA, PCA and Water.

Every centrifugate of each run described in Table I was made up to 100 ml and D_{260} and P_t were estimated.

—○— D_{260} —●— P_t

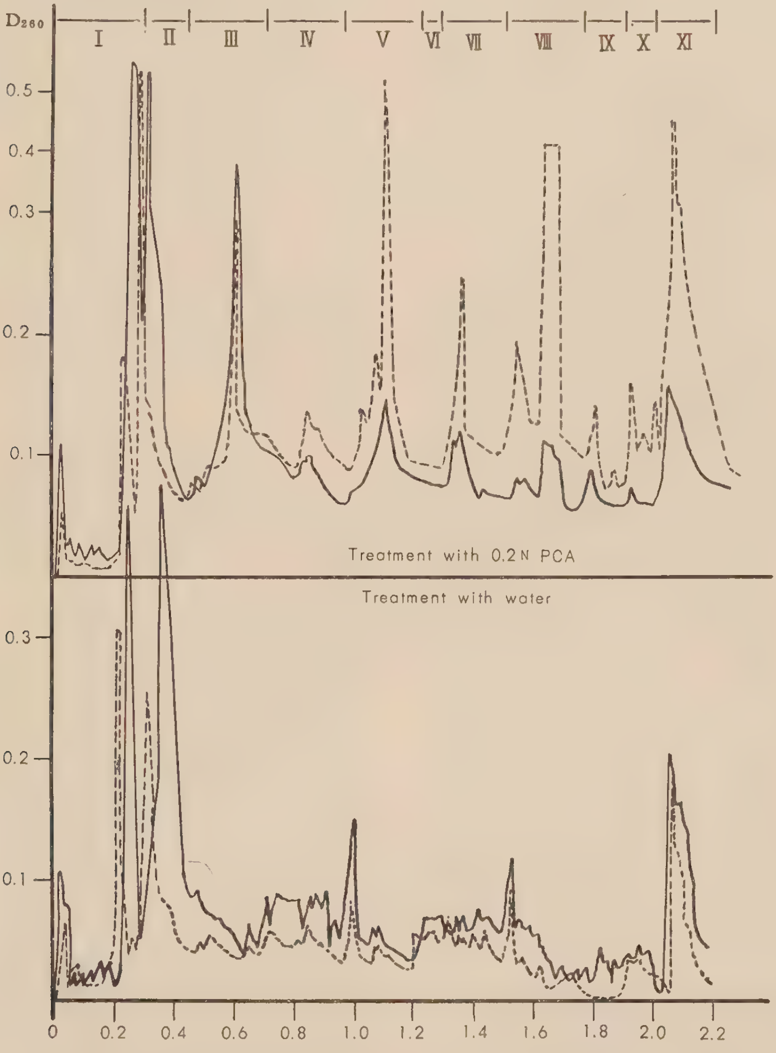

FIG. 3. Degradation of RNA and its Dissolution in the Acid-Soluble Fraction when Acid-Soluble Compounds were extracted repeatedly with PCA.

Acid-soluble compounds were extracted fifteen times with 0.2 N PCA or water. The centrifugates ranging from the first to seventh extraction (full line) and the eighth to fifteenth extraction (dotted line) were separately united and each mixture was analysed by ion exchange chromatography.

Ion exchanger: Dowex-1-chloride, 200~400 mesh, 27 cm×2.3 cm^2

Eluting solution: I; 0.005 N HCl, II; 0.01 N HCl, III~IX; 0.01 N HCl+0.0125 N, 0.025 N, 0.05 N, 0.1 N, 0.2 N, 0.4 N and 1 N NaCl, X; 0.1 N HCl, XI; 2 N HCl.

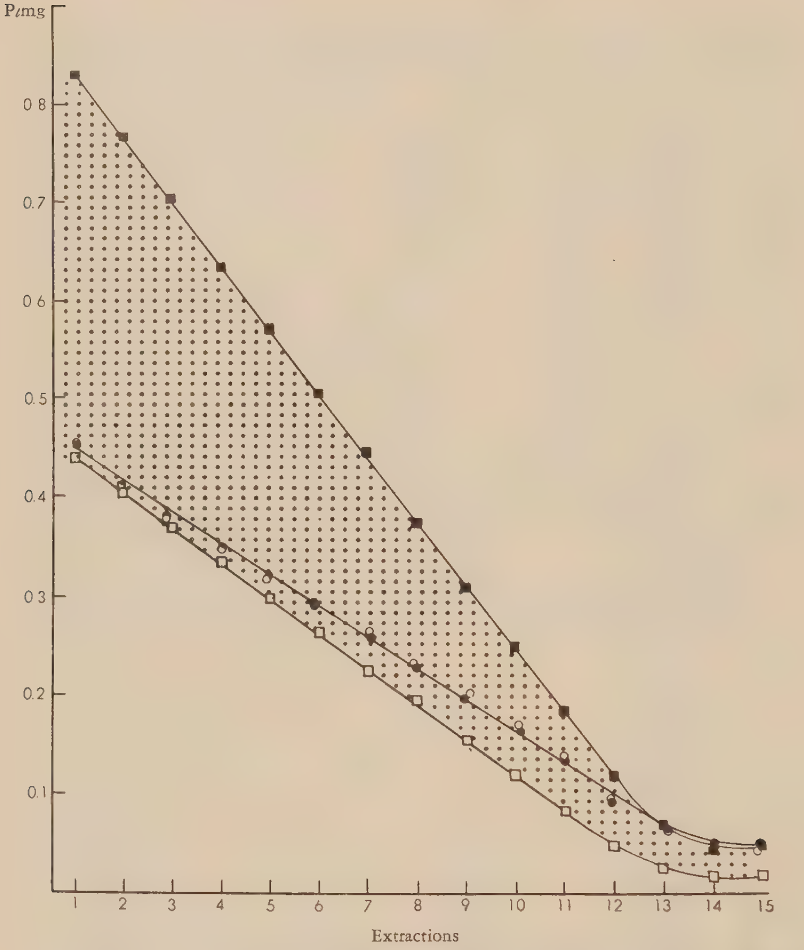

FIG. 4. Dissolution of Acid-Soluble Compounds into the RNA Fraction, on the Removal of Acid-Soluble Compounds with PCA at 0°C.

Residual yeast used for the extraction of RNA was pretreated with 0.2 N PCA or water for removal of acid-soluble compounds. They were allowed to stand overnight in 5 ml of 1 N PCA at 0°C, and then extracted fourteen times with 5 ml of 1 N PCA for 30 min. each time in the cold. The extraction of RNA was investigated by the estimation of D_{260} and P_t.

—○— RNAP, when extracted with water.
—□— RNAP, when extracted with PCA.
—●— P_t, when extracted with water.
—■— P_t, when extracted with PCA.

PCA.

If acid-soluble compounds could be extracted completely, P_t would be equal to RNAP in the RNA fraction. It has been apparently recognized as seen in Table I that acid-soluble compounds are incompletely extracted with TCA or PCA under the conditions described in Table I, because P_t was always larger than RNAP in the RNA fraction. Only when the frozen yeast was extracted with water in place of with PCA or TCA, P_t and RNAP agreed well with each other. This is a very important and interesting fact.

If RNAP in the RNA fraction is measured spectrophotometrically, the effect of the pretreatment at 0°C with either water, TCA or PCA on RNAP seems to be within experimental error, because the amount of acid-soluble fraction estimated spectrophotometrically is much smaller than that of RNA. However, when one attempts to calculate RNAP from phosphorus content, the result becomes serious, because Ogur and Rosen's method consists of the fact that RNAP value measured spectrophotometrically coincides with that calculated from phosphorus.

In order to check the extraction of acid-soluble compounds in detail, D_{260} and P_t were chased in every centrifugate of each run described in Table I and the results summarized in Fig. 2. A few times' repetition of the extraction, prescribed by Ogur and Rosen's method, of acid-soluble compounds with 0.2 N PCA was so insufficient that this may lead to erroneous estimation of RNA. However, even when the extraction is repeated further with the above-mentioned solvents, acid-soluble compounds could not be extracted completely ; on the contrary, dissolution of RNA into the acid-soluble fraction was observed as described below; the values of D_{260} decreased gradually and then began to increase after about the fifth extraction. This fact may be caused by the degradation of RNA.

When water, in place of PCA or TCA, was employed for the extraction, acid-soluble compounds could be removed completely without any loss of RNA even at 10°C.

In order to demonstrate the degradation and the dissolution of RNA, acid-soluble compounds extracted with water and with PCA were compared by ion-exchange chromatography. Attempts were not made to identify each peak of the elution diagram in Fig. 3, but nucleotides ouget to be eluted in part II to XI[9]. In the case of the extraction with PCA, the amount of nucleotides contained in the combined centrifugate of the eighth to fifteenth extraction was larger than that in the combined centrifugate of the first to seventh extraction. This peculiar phenomenon could not be recognized by the use of the extraction with water.

Therefore, it would be reasonably considered in Table I that the small decrease in amount of RNAP on the extraction with TCA or PCA, might be due to the degradation of RNA to produce acid-soluble phosphorus.

From these results, it is desirable that acid-soluble compounds should be extracted with water ten times at a temperature below 10°C.

3) Extraction of RNA.

For the extraction of RNA from the sample free from alcohol-ether-soluble and acid-soluble compounds, Ogur and Rosen's method prescribes in the case of root tips or pollen cells that the sample should be stored in 1 N PCA for eighteen hours in the cold and then extraction twice more.

Effect of the pretreatment for the removal of acid-soluble compounds on the extraction of RNA was studied. For this purpose, the residual yeast which was pretreated with water or 0.2 N PCA for the removal of acid-soluble compounds, was extracted as shown in Fig. 4. The extractability of RNA was chased by the estimation of D_{260} and P_t in each centrifugate. It was recognized, as shown in Fig. 4 that when RNA was extracted from the residue treated with 0.2 N PCA, some part of the acid-soluble compounds was found in the RNA fraction in consequence of the insufficient extraction with 0.2 N PCA. The amount of contaminating acid-soluble compounds is shown by the dotted area, but these compounds do not seem to have absorption near 260 mμ. On the other hand, only when the yeast was pretreated with water, RNA free from acid-soluble compounds was obtained. This can be seen from the fact that the RNA value estimated from the optical density at 260 mμ agreed with that obtained from the estimation of P_t in this fraction.

Fig. 4 also shows that RNA was sufficiently extracted after fifteen extractions with 1 N PCA. RNA fraction thus obtained was considered to be free from DNA, because diphenylamine reaction was negative.

4) Extraction of DNA.

According to Ogur and Rosen's method, DNA was extracted three times with 5 ml of 0.5 N PCA at 70°C for twenty minutes from the residual yeast after the extraction of RNA in experiment 3).

The centrifugates were combined and the value of DNA calculated from the determination of optical density at 268 mμ and that obtained by the colorimetric determination with diphenylamine were compared. A good agreement was observed between them, even when different solvents were used for the extraction of

TABLE II. EFFECT OF DIFFERENT PRETREATMENTS FOR THE REMOVAL OF ACID-SOLUBLE COMPOUNDS ON DNA CONTENT.

The residual yeast remained after the extraction of RNA in experiment 3) was extracted at 70°C in 5 ml of 0.5 N PCA, three times. Three extracts were united and DNAP and P_t estimated.

Yeast residue after extraction RNA in Fig. 4	DNAP		P_t
	D_{268}	diphenylamine	
	mg	mg	mg
Removal of acid-soluble compds. with water	0.10	0.11	0.24
Removal of acid-soluble compds. with 0.2 N PCA	0.10	0.10	0.23

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9) E. Volkin and W.E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).

acid-soluble compounds, as shown in Table II.

However, P_t was appreciably larger than DNAP. This phenomenon appears to be due to the contamination with phosphoprotein ; this fact is well demonstrated by the fact that the higher the extracting temperature, the more phosphorus was extracted and the less the phosphoprotein remained. This will be further discussed in a following paper[10]. Hence, in the determination of DNA, diphenylamine reaction is preferred to spectrophotometric measurement.

DISCUSSION

All the experiments described above were studied on yeast. Acid-soluble compounds were extracted incompletely with PCA or TCA, both from fresh and frozen yeast.

However, it is worth-while to note that acid-soluble compounds could be extracted completely, only when the frozen yeast was extracted with water. Such a phenomenon was also experienced by the present authors in the case of the isolation of thiamine[11] or glutathione[12,13] from yeast, though these substances are apparently soluble in acid. This is considered to be due to the permeability of yeast cell membrane.

From this point of view, the following two experiments were undertaken. One experiment was on the sample of which cell membrane was easily destroyed in a mortar, for example, rabbit liver. In this case, the acid-soluble compounds were easily extracted with TCA or PCA, their dissolution into the RNA fraction being not recognized. The other was on the effect of destruction of the yeast cell membrane with sonic vibration. Two grams of pressed yeast was sonated at 10 Kc. in 20 ml of ethanol for one hour. Measurement of the acid-soluble compounds and RNA after this treatment resulted in agreement with the values obtained from the yeast pretreated of freezing, as shown in Table III.

TABLE III. EXTRACTION OF ACID-SOLUBLE COMPOUNDS AND RNA FROM THE YEAST AFTER FREEZING OR SONATION.

Two grams of pressed yeast was sonated at 10 Kc. in 20 ml of ethanol for 1 hr. or frozen with dry ice and ether. Acid-soluble compounds and RNA were extracted by the proposed procedure respectively.

Pretreatment	Acid-soluble fraction P_t mg	RNA fraction PNAP mg
Freezing with dry ice and ether	8.04	1.89
Sonation	8.04	1.89

Therefore, the peculiar extraction of acid-soluble compounds from fresh, durable or frozen yeast with TCA or PCA, may be due to the permeability of yeast cell membrane which is not easily destroyed mechanically.

SUMMARY

1) In the case of yeast, it has been demonstrated that acid-soluble compounds are not sufficiently eliminated when extracted twice with 0.2 N PCA according to Ogur and Rosen's method.

2) Even if the extraction with 0.2 N PCA is repeated further, acid-soluble compounds are not extracted completely ; on the contrary, RNA begins to dissolve into the acid-soluble fraction.

3) It has been shown that these defects can be eliminated only when acid-soluble compounds are extracted with water instead of with 0.2 N PCA.

4) Based on the facts described above, the previous report on the determination of nucleic acids in fresh or durable yeast has been further modified, from the viewpoint of elution behavior of acid-soluble compounds.

Acknowledgement The authors would like to thank Messrs. M. Tokikuni and T. Kumazawa, the managing directors of Kirin Brewery Co. Ltd., who gave us the opportunity to publish this papar. We are grateful for many suggestions and the continued interest of Dr. Y. Umeda, the president of the Research Laboratories of Kirin Brewery Co. Ltd., and Dr. Y. Okuda, a member of the Japan Academy and the former president of our Research Laboratories.

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10) Y. Kuroiwa and K. Nakagawa, in press.

11) Y. Kuroiwa, unpublished.

12) Y. Kuroiwa, U. Ito, H. Horiguchi and S. Shimamoto, *J. Japan Soc. Food and Nutrition*, **2**, 119 (1949).

13) Y. Kuroiwa, *J. Japan Soc. Food and Nutrition*, **2**, 143 (1949).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 555~557, 1960]

Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

Part VI. Basic Properties of Each Crystalline Component

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Received February 29, 1960

Crystalline yeast phosphoglyceric acid mutase is composed of several components. Though they have the same crystalline forms, the differences are found among their specific activities. Hereupon, it is necessary to elucidate the enzymatic properties of these components. As the results, the basic properties of each crystalline component have been found to be the same.

INTRODUCTION

Crystalline yeast PGA mutase* is found to be composed of several components by means of electrophoretic analysis[1~3].

As already reported[3], we constructed the special vertical zone electrophoresis apparatus and we succeeded in the separation of large amounts of each component and in the crystallization of them. Though these components have different specific activities, they have the same crystalline forms. Moreover, their crystalline forms are all the same as that of the original crystalline enzyme. From these results we suggested that there was no essential difference among the proteins of these components. In order to elucidate the fundamental problem why the specific activity differs from each other, it is necessary to investigate the differences among the enzymological properties of each component. If each component has different values of apparent Kc for the coenzyme, of Keq of the PGA mutase reaction and of optimal pH, the specific activity must differ from each other. But we have not found any difference among their enzymological properties. In this paper, the basic properties of each crystalline component are described.

MATERIALS AND METHODS

Each component in the original crystalline enzyme was separated by means of vertical zone electrophoresis and crystallized as described in the previous paper[3].

DL-2PGA (substrate) and 2,3-PGA (coenzyme) were prepared by the methods described in the previous paper[4]. Barium salt of D-3PGA was obtained from the Nutritional Biochemicals Corporation.

The assay conditions of the enzyme activity were the same as described before[4].

Protein concentrations were determined spectrophotometrically by measuring the light absorption at 280 mμ as described before[4].

RESULTS AND DISCUSSION

Apparent Michaelis Constant for the Coenzyme. As described previously, yeast PGA mutase is inhibited by the substrate, because the substrate is a competitive inhibitor of the coenzyme[5].

Since the apparent Michaelis constant for the coenzyme (Kc′) is dependent on the substrate concentrations, we must compare the Kc′ values of each component which were determined under the same conditions.

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* The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; DL-2PGA, DL-2-phosphoglyceric acid; D-3PGA, D-3-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; coenzyme, 2,3-PGA.

[1] H. Edelhoch, V.W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

[2] H. Chiba and E. Sugimoto, This Bulletin, **23**, 213 (1959).

[3] H. Chiba, E. Sugimoto and M. Kito, This Bulletin, **24**, 428 (1960).

[4] H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959).

[5] H. Chiba, E. Sugimoto and M. Kito, This Bulletin, **24**, 418 (1960).

TABLE I. BASIC PROPERTIES OF EACH CRYSTALLINE COMPONENT

Component	Specific Activity	Kc′	Keq (3PGA/2PGA)	pH optimum	$E^{1\%}_{1\mathrm{cm}}$ (280 mμ)	$E_{280\mathrm{m}\mu}/E_{260\mathrm{m}\mu}$
Original crystal	1,800	3.31×10^{-4}M	5.2	5.9	13.8	1.97
I	4,060	3.31× 〃	5.2	5.9	14.0	1.91
II	3,030	3.33× 〃	5.1	5.9	13.9	1.98
III	2,240	3.30× 〃	5.3	5.9	14.1	1.93
IV	200	3.31× 〃	5.2	5.9	14.2	1.85

In the case of the determination of Kc′ of each component, the final concentration of the substrate under the conditions of the standard assay[4)] was used. The conditions of the reaction were the same as those for the standard assay except that the coenzyme concentrations were varied. The final concentration of DL-2 PGA was 1.65×10^{-2} M, and the reaction mixture was incubated at 25°C for ten minutes. The reaction rate was directly determined by the polarimetric method[4)]. The coenzyme was given within the range of 5×10^{-5} to 10^{-3} M.

In these experiments, the amounts of each component used were as follows: component I, 4.5 μg; II, 7.3 μg; III, 10.3 μg; and IV, 94.0 μg. The Kc′ values determined under the same conditions are given in Table I.

It is clearly found that the Kc′ values of each component determined under the same substrate concentration are the very same. From these facts, it is revealed that each component is inhibited by the substrate to the same degree and that there is no difference among the affinities of each component to the coenzyme.

As the Kc′ values of each component are 3.31×10^{-4} M, it is natural that the original crystalline enzyme containing each component has the Kc′ value of 3.31×10^{-4} M.

Equilibrium Constant at 25°C and pH 5.9. The equilibrium constants of the enzyme reactions catalyzed by each component were determined with D-3 PGA as the substrate (25°C, pH 5.9). The conditions of the reaction were the same as those for the Keq assay in the previous paper[4)].

The reaction mixture contained (in micromoles) D-3 PGA, 137; the coenzyme, 0.25; acetate buffer, 150; and adequate amounts of each component. In these experiments, the amounts of each component used were as follows: component I, 55.0 μg; II, 92.5 μg; III, 153.3 μg; and IV, 850.0 μg.

As shown in Table I, Keq values determined under the same conditions of the reaction are the same.

pH Optimum. The effects of pH on the PGA mutase activities of each component were investigated. The conditions of the reaction were the same as those for the standard assay except that pH values of the reaction mixtures were varied.

The pH-activity relationship of each component was studied over the range of pH 4.5 to 8.0. In these experiments, the amounts of each component used were as follows: component I, 4.5 μg; II, 7.3 μg; III, 10.3 μg; and IV, 94.0 μg.

As shown in Table I, the optimal pH values of each component are the same (pH 5.9).

$E^{1\%}_{1\mathrm{cm}}$ (280 mμ) and $E_{280\mathrm{m}\mu}/E_{260\mathrm{m}\mu}$. After each crystalline component was dialysed against distilled water for a few days, its dry weight was determined. $E^{1\%}_{1\mathrm{cm}}$ (280 mμ) values of each component were calculated. These results are given in Table I.

It is obvious that $E^{1\%}_{1\mathrm{cm}}$ (280 mμ) values of each component are identical and $E_{280\mathrm{m}\mu}/E_{260\mathrm{m}\mu}$ absorption ratios are also similar.

As clearly shown in the results described above, there is no difference among the basic properties of each component.

Besides, as reported previously, the crystalline forms of the individual components are the same[3)], and they all reveal low activities without the addition of the coenzyme, and their behaviors in the fluoride inhibition are also similar[2].

Hereupon, the individual components may be regarded as almost the same enzyme protein except for the differences among their specific activities and mobilities.

As clearly shown in the following paper[6], in the original crystalline enzyme obtained from the autolysate incubated for 15 hours (15 hr.-crystal), component I disappears and component II, III, IV, V and VI are present.

Therefore, since in this and previous papers[3] the 15 hr.-crystal was used as the original crystal, component I should be corrected to component II, II to III, III to IV, IV to V and V to VI.

SUMMARY

The apparent Michaelis constants of each component for the coenzyme are the same values.

Each component is inhibited by the substrate according to the same mechanism.

The optimal pH values and the equilibrium constants of the reactions catalyzed by each component are the same.

$E_{1\,\mathrm{cm}}^{1\%}$ (280 mμ) values of each component are are identical. $E_{280\,\mathrm{m}\mu}/E_{260\,\mathrm{m}\mu}$ absorption ratios also similar.

It may be concluded that each component is almost the same enzyme protein.

Acknowledgement The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice throughout the course of this work.

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[6] H. Chiba, E. Sugimoto and M. Kito, This Bulletin, **24**, 558 (1960).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 558~561, 1960]

Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

Part VII. Crystalline Preparations from Autolysates Incubated for Various Periods

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It has been found that the crystalline phosphoglyceric acid mutase preparations obtained from the autolysates incubated for different periods have different specific activities, electrophoretic patterns and proportions of components which construct the crystalline enzyme, though these crystals have the same crystalline forms. Consequently, it is concluded that the yeast phosphoglyceric acid mutase protein is component I in native state in yeast cells and component I is attacked and modified into the other components by PGA mutase-modifying enzyme in yeast.

INTRODUCTION

It is interesting to study what relations exist among the several components involved in the original crystal of yeast PGA mutase[*][1~3].

There occurs a question whether the several components exist originally as the native proteins in yeast cells or not. Namely, it is probable that the enzyme protein which is originally one component is modified during the isolation and purification procedures of PGA mutase from baker's yeast.

We, however, have obtained the crystalline preparation with the very low specific activity from the autolysate of baker's yeast, when the autolysis time is prolonged. This fact suggests that the PGA mutase protein is modified and inactivated by PGA mutasemodifying enzyme in yeast cells during autolysis.

In this respect, we have varied the autolysis time and obtained the various crystalline PGA mutase preparations. Hereupon, we have been able to study the differences of the crystalline forms, the specific activities and the electrophoretic behaviors among these crystalline preparations.

In this paper, these investigations are described.

MATERIALS AND METHODS

The three times recrystallized PGA mutase preparations were obtained as described in the previous paper[4], but in the purification procedures different autolysis time was chosen.

DL-2PGA and 2,3-PGA were prepared by the method described in the previous paper[4].

The assay conditions of the enzyme activity were the same as described before[4].

Protein concentrations were determined by measuring the absorption at 280 mμ in a Beckman DU spectrophotometer, with an extinction coefficient $E_{1cm}^{1\%}$ 13.8 for crystalline yeast PGA mutase[4].

Boundary electrophoretic analysis was performed at 0.7°C with a Tiselius apparatus equipped with a diagonal slit device of Svenson[5].

Nitrogen was determined by means of micro-Kjeldahl method.

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[*] The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; DL-2PGA, DL-2-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; coenzyme, 2,3-PGA.

[1] H. Edelhoch, V.W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

[2] H. Chiba and E. Sugimoto, This Bulletin, **23**, 213 (1959).

[3] H. Chiba, E. Sugimoto and M. Kito, This Bulletin, **24**, 428 (1960).

[4] H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959).

[5] H. Svenson, *Kolloid. Z.*, **87**, 181 (1939).

RESULTS AND DISCUSSION

Relation between the Enzyme Activity of the Water Extract and the Autolysis Time. Baker's yeast was incubated with toluene and then the PGA mutase activities of the water extracts from the autolysates were studied in relation to the autolysis time.

Fragmented, fresh baker's yeast was mixed with one-tenth quantity of toluene at 38°C. The liquefied mixture was left to stand for several hours at 38°C. After the adequate autolysis time, 50 ml of cold distilled water was added to 50 ml of the autolysate and the mixture was cooled to 0°C, and then the extract was obtained as a clear solution by centrifugation (50,000×g, 15 min., at 0°C).

The relation between the enzyme activities of the extracts and different autolysis time is shown in Table I.

TABLE I. RELATION BETWEEN ENZYME ACTIVITY OF EXTRACT AND AUTOLYSIS TIME

Autolysis time	Water extract		
	Enzyme Unit/ml	N/ml	Enzyme Unit/mg of N
hrs.		mg	
2.0	490	2.64	185
3.0	740	3.00	246
4.5	930	3.46	268
6.0	1,060	3.87	274
7.5	1,220	4.27	286
9.0	1,250	4.68	268
10.5	1,310	5.10	257
12.0	1,310	5.27	249
13.5	1,220	5.50	222
15.0	950	5.78	164
17.0	650	6.00	108
20.0	190	6.40	30
24.0	68	6.85	10

From these results, it is obvious that though the amounts of nitrogeneous substances increase in proportion to the autolysis time, the enzyme activities of the extracts increase till 12 hours of the autolysis time, and then decrease. The enzyme activities per mg of nitrogen reveal a similar tendency.

These facts suggest that the PGA mutase protein is attacked and inactivated by PGA mutase-modifying enzyme in some way during autolysis of yeast.

If the yeast PGA mutase protein is modified by PGA mutase-modifying enzyme in yeast cells during autolysis, it is expected that the crystalline enzyme preparations obtained from autolysates have different specific activities. Therefore, it is necessary for further investigations to prepare crystalline PGA mutase from baker's yeast incubated for different periods.

Crystalline Preparations Obtained from Autolysates Incubated for Different Periods. According to the method described before[4)], we succeeded in preparing several kinds of the crystalline enzyme preparations with various specific activities.

In these experiments, the autolysis time is changed as follows, 3, 6, 9, 12, 15 and 20 hours (38°C). We used 8 pounds of baker's yeast as starting material for preparing the crystalline enzyme preparations in cases of 3 and 6 hours' autolysis, and 4 pounds in cases of 9, 12, 15 and 20 hours' autolysis.

In spite of the variation of the autolysis time, the crystalline forms of these crystalline preparations, are the same (rhombic plate). The specific activities of these crystalline preparations however, are different from each other as shown in Table II.

TABLE II. SPECIFIC ACTIVITIES OF CRYSTALS OBTAINED FROM AUTOLYSATES INCUBATED FOR DIFFERENT PERIODS

Crystals	Specific Activity
3 hr.-crystal	4,010
6 hr.-crystal	3,350
9 hr.-crystal	2,730
12 hr.-crystal	2,460
15 hr.-crystal	1,850
20 hr.-crystal	190

The specific activity decreases as the autolysis time is prolonged. These results agree with those expected in Table I. Thence, it is the characteristic of yeast PGA mutase that these crystals have the same crystalline forms, notwithstanding having different specific activities.

As described previously[3)], from the fact that the crystalline form of each component separated

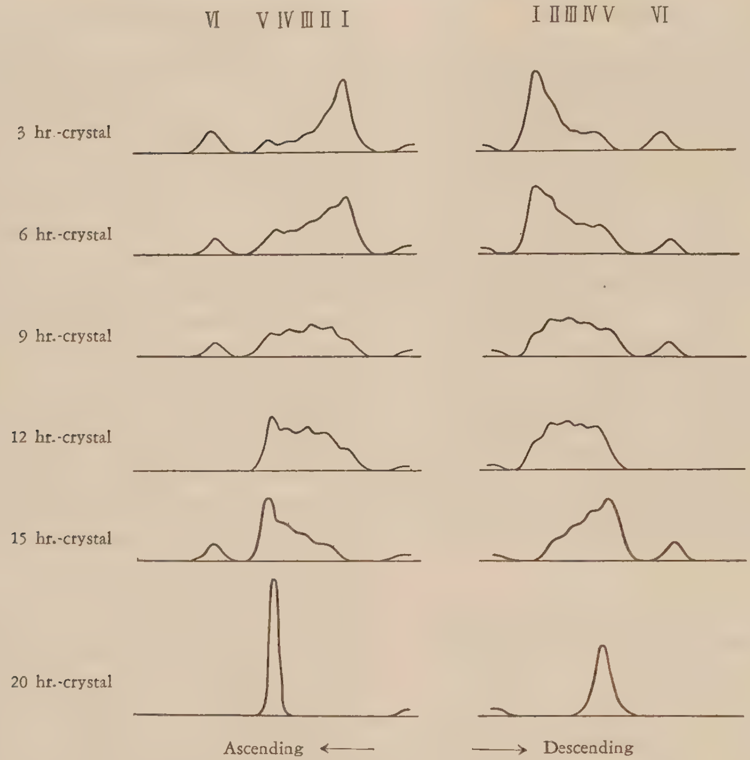

FIG. 1. Boundary Electrophoretic Patterns of Various Crystalline Preparations.

The patterns were obtained from about 2 per cent protein solutions of the various crystals in a veronal buffer of pH 7.0 and 0.068 ionic strength at a potential gradient of 4.05 volts per cm for 14,400 seconds (0.7°C). The numbers referred to each component.

by vertical zone electrophoresis is the same (rhombic plate), the results described above can be easily understood.

In Fig. 1, the electrophoretic patterns of these crystals obtained under the different conditions are shown. The electrophoretic experiments were carried out at 0.7°C in a veronal buffer at pH 7.0 and ionic strength 0.068 at a potential gradient of 4.05 volts per cm for 14,400 seconds.

It is clearly shown that several components exist in different proportions in these crystals.

The crystalline enzyme obtained from the autolysate incubated for 3 hours at 38°C (3 hr.-crystal) has the highest content of component I, and then the content of this component in the other crystals decreases as the autolysis time is prolonged.

As described before[2,3], component I with the slowest mobility has the highest specific activity, and the specific activities of the other components become lower in order. Since component VI has no PGA mutase activity, this component is an impurity, and can be removed by many times recrystallizations. Therefore, sometimes, this component exists in the crystalline preparations.

In the crystals with the lower specific activities (6, 9, 12, 15 and 20 hr.-crystals) the content of component V with the lowest specific activity increases in proportion to the autolysis time.

When we compare the electrophoretic patterns of the 3 and 6 hr.-crystals with those of the 12 and 15 hr.-crystals, the ratios of the individual components are found to be inverse. In the

15 hr.-crystal, component I disappeares. In the 20 hr.-crystal, component I, II, III and IV have vanished, and only component V remains. Therefore, the specific activity of the 20 hr.-crystal becomes extremely low.

It is evident from the facts described above that component I is turned into component II, III, IV and V as the autolysis time is prolonged.

These facts show that the yeast PGA mutase protein exists as a single component, namely component I, in native state in yeast cells and during autolysis component I is attacked and modified into the other components by PGA mutase-modifying enzyme in yeast.

In order to elucidate the reaction mechanism of this PGA mutase-modifying enzyme, we are investigating the properties of this modifying enzyme.

When specific inhibitors for this modifying enzyme are found, we may be able to isolate crystalline PGA mutase of only componet I from baker's yeast.

SUMMARY

The relation between the enzyme activity of the watar extract and the autolysis time is investigated.

The crystalline PGA mutase preparations having the same crystalline forms and different specific activities are obtained from the autolysates incubated for different periods at 38°C.

The specific activities of these crystals decrease as the autolysis time is prolonged.

The content of component I in these crystals decreases in proportion to the autolysis time.

The 20 hr.-crystal is component V only.

The PGA mutase protein is only component I in yeast cells and this component is modified by PGA mutase-modifying enzyme during autolysis.

Acknowledgement The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice throughout the course of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 562~564, 1960]

Studies on the Degradation Mechanisms of Proteins and Their Derivatives in the Foods

Part IV. The Decompositions of Several Mercaptals and Mercaptoles of Cysteine in Aqueous Solutions

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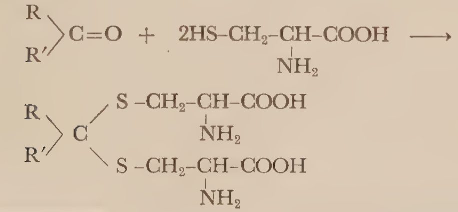
Received March 9, 1960

Cysteine mercaptals and mercaptoles were prepared by the reactions of L-cysteine with formaldehyde, acetaldehyde, *n*-butyraldehyde, benzaldehyde, furfural, pyruvic acid and levulinic acid in 6 N hydrochloric or sulfuric acid. Hydrogen sulfide released from cysteine mercaptals and mercaptoles in heated aqueous solutions (oil bath: 120°C) was determined. Although a small amount of hydrogen sulfide was liberated from L-cystine on one hour heating, its amount increased suddenly after three hours. Among these compounds L-cysteine mercaptal of furfural was most unstable and a large amount of hydrogen sulfide was produced.

In the previous paper[1] several thiazolidine derivatives were prepared, and hydrogen sulfide and ammonia released from them in heated aqueous solutions were determined. It has been shown that thiazolidine derivatives are stable generally in boiling aqueous solutions, but L-cysteine-furfural compound is unstable and a large amount of hydrogen sulfide compared with other compounds is released.

In the present paper several L-cysteine mercaptals and mercaptoles were prepared from L-cysteine and aldehydes (formaldehyde, acetaldehyde, *n*-butyraldehyde, benzaldehyde and furfural) or ketonic acids (pyruvic acid and levulinic acid) in 6 N hydrochloric or sulfuric acid[2].



$$\begin{matrix}R\\R'\end{matrix}\!\!>C=O + 2HS\text{-}CH_2\text{-}\underset{NH_2}{CH}\text{-}COOH \longrightarrow$$

$$\begin{matrix}R\\R'\end{matrix}\!\!>C\begin{matrix}\diagup S\text{-}CH_2\text{-}\underset{NH_2}{CH}\text{-}COOH\\ \diagdown S\text{-}CH_2\text{-}\underset{NH_2}{CH}\text{-}COOH\end{matrix}$$

Hydrogen sulfide released from these compounds in aqueous solutions heated on the oil bath (120°C) was determined in the same manner as described in the previous paper[1].

The following results were obtained. The smallest amount of hydrogen sulfide among these compounds was liberated from L-cystine on one hour heating, but its amount increased suddenly after three hours. Among these compounds L-cysteine mercaptal of furfural was most unstable and a large amount of hydrogen sulfide was released. L-Cysteine mercaptals of formaldehyde, acetaldehyde and *n*-butyraldehyde were stable comparatively.

EXPERIMENTAL

1. L-Cysteine.

L-Cysteine was prepared by the reduction of L-cystine, which was separated from human hair, with tin-hydrochloric acid.

2. Mercaptals and mercaptoles of L-cysteine.

Djenkolic acid, and L-cysteine mercaptals of acetaldehyde and benzaldehyde were prepared by the method of Armstrong and du Vigneaud[2]. L-Cysteine mercaptals of *n*-butyraldehyde and furfural, and L-cysteine mercaptoles of pyruvic acid and levulinic acid were also obtained similarly.

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[1] J. Mizutani, Y. Obata and Y. Ishikawa, This Bulletin, in press.

[2] M.D. Armstrong and V. du Vigneaud, *J. Biol. Chem.*, **168**, 373~7 (1947); *ibid.*, **173**, 749~751 (1948).

Djenkolic acid monohydrochloride. L-Cysteine (16.6 m mol) was dissolved in 4 ml of 6N hydrochloric acid, 37% formaldehyde (8.3 m mol) added, and the resulting solution was warmed to 70°C. It was then allowed to cool to room temperature and stand overnight. It was neutralized by the addition of 6N sodium hydroxide and the precipitated djenkolic acid was collected and washed with water. The monohydrochloride was obtained by dissolving djenkolic acid in hot 1% hydrochloric acid and cooling the solution; yielded 1.2 g (50 %). It was recrystallized from 1 % hydrochloric acid, m.p. 250~5°C (dec.).

Nitrogen was determined by the method of semimicro-Kjeldahl[3] (decomposition agents: concentrated sulfuric acid, potassium sulfate and mercuric oxide), sulfur by alkali fusion[4], and chlorine was determined by gravimetric analysis[5] (AgCl).

Anal. Found: N, 9.30; S, 22.52; Cl, 11.89 %.
Calcd. for $C_7H_{15}N_2O_4S_2Cl$: N, 9.64; S, 22.05; Cl, 12.19 %.

L-Cysteine mercaptal of acetaldehyde monohydrochloride. To a solution of L-cysteine (16.6 m mol) in 4 ml of 6 N hydrochloric acid was added 92% acetaldehyde (8.3 m mol). The mixture was shaken for a while and allowed to stand for one hour at room temperature. After keeping in an ice-box overnight, crystals were collected. They were recrystallized from 50% ethanol; yielded 1.1 g (44%), m.p. 225~7°C (dec.), reported[2] m.p. 205~230°C (dec.).

Anal. Found: N, 9.59; S, 20.90; Cl, 11.50 %.
Calcd. for $C_8H_{17}N_2O_4S_2Cl$: N, 9.19; S, 21.04; Cl, 11.63 %.

L-Cysteine mercaptal of *n*-butyraldehyde monohydrochloride. L-Cysteine mercaptal of *n*-butyraldehyde monohydrochloride was prepared from L-cysteine (16.6 m mol) and *n*-butyraldehyde (8.3 m mol) in 4 ml of 6 N hydrochloric acid in the same manner as the corresponding compound from acetaldehyde; yielded 1.4 g (51%). m.p. 213~5°C (dec.).

Anal. Found: N, 8.52; S, 19.76; Cl, 10.55 %.
Calcd. for $C_{10}H_{21}N_2O_4S_2Cl$: N, 8.42; S, 19.27; Cl, 10.65 %.

L-Cysteine mercaptal of benzaldehyde. To a solution of L-cysteine (16.6 m mol) in 4 ml of 6 N hydrochloric acid was added benzaldehyde (8.3 m mol) and the mixture shaken occasionally. After standing at room temperature for an hour, the clear solution was diluted with an equal volume of water, shaken with ether to remove benzaldehyde which did not react, and the solution was neutralized with 6 N sodium hydroxide. The resulting gelatinous precipitate was recrystallized from hot water; yielded 1.6 g (59 %) and m.p. 208~210°C (dec.); reported[2] m.p. 200~220°C (dec.).

Anal. Found: N, 8.23; S, 19.70 %.
Calcd. for $C_{13}H_{18}N_2O_4S_2$: N, 8.48; S, 19.41 %.

L-Cysteine mercaptal of furfural. L-Cysteine (16.6 m mol) was dissolved in 4 ml of 6 N sulfuric acid, furfural (8.3 m mol) added and the mixture was shaken at intervals. After standing at room temperature for an hour, the solution was made neutral by the addition of 6 N sodium hydroxide. The resulting precipitate was dissolved in hot water, the solution decolorized with active carbon and crystals were obtained by cooling; yielded 1.5 g (57 %). They were recrystallized from hot water, m.p. 195~200°C (dec.).

Anal. Found: N, 8.24; S, 20.16 %.
Calcd. for $C_{11}H_{16}N_2O_5S_2$: N, 8.75; S, 20.02 %.

L-Cysteine mercaptole of pyruvic acid monohydrochloride. To a solution of L-cysteine (16.6 m mol) in 4 ml of 6 N hydrochloric acid was added pyruvic acid (8.3 m mol), the mixture was warmed to 70°C, and cooled to room temperature. After standing overnight, the solution was evaporated to almost dryness under reduced pressure. The residue was taken in ethanol and precipitated by the addition of ether. The yield was 1.3 g (45 %) and the compound had no constant melting point.

Anal. Found: N, 7.61; S, 19.62 %.
Calcd. for $C_9H_{17}N_2O_6S_2Cl$: N, 8.03; S, 18.38 %.

This compound gave a positive test for the sulfhydryl group when treated with sodium nitroprusside.

L-Cysteine mercaptole of levulinic acid monohydrochloride. This compound was prepared from L-cysteine (16.6 m mol) and levulinic acid (8.3 m mol) in 4 ml of 6 N hydrochloric acid in the same manner as L-cysteine mercaptole of pyruvic acid monohydrochloride; yielded 1.6 g (52 %).

Anal. Found: N, 7.18; S, 17.07 %.
Calcd. for $C_{11}H_{21}N_2O_6S_2Cl$: N, 7.43; S,

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[3] S. Akabori and S. Mizushima, "Chemistry of Protein", Kyoritsu Publishing Co., Ltd., (1) p. 109 (1954); J.B. Sumner and G.F. Somers, "Laboratory Experiments in Biological Chemistry," 1949, p. 67~71; Dept. of Agr. Chem., Kyoto Univ., "Laboratory Experiments in Agricultural Chemistry," 1957, p. 134~5.

[4] K. Bailey, *Biochem. J.*, **31**, 1398 (1937).

[5] Dept. of Agr. Chem., Kyoto Univ., "Laboratory Experiments in Agricultural Chemistry," 1957, p. 104.

17.02 %.

3. Determination of hydrogen sulfide.

Hydrogen sulfide released from cysteine mercaptals and mercaptoles in heated aqueous solutions was determined in the same manner as described in the previous paper[1]. However, although in the previous paper to a sample containing 32 mg of sulfur were added 10 ml of water, in the present paper 100 ml of water were added in order to dissolve cystine completely. As the result of it the amounts of hydrogen sulfide from cystine and cysteine were increased considerably as compared with the values in the previous paper. After dissolving in 100 ml of water, hydrochlorides were neutralized with 6 N sodium hydroxide. Carbon dioxide gas current was used as a carrier of hydrogen sulfide. After heating for one, three and six hours respectively on the oil bath (120°C), to the reaction mixture were added 20 ml of 6 N hydrochloric acid and carbon dioxide was passed through the solution furthermore thirty minutes. Hydrogen sulfide absorbed in 2% zinc acetate was derived into methylene blue and the absorbancy at 745 mμ was determined spectrophotometrically.

The results of them are shown in Table I.

TABLE I. DETERMINATIONS OF HYDROGEN SULFIDE LIBERATED FROM L-CYSTEINE MERCAPTALS AND MERCAPTOLS IN HEATED AQUEOUS SOLUTIONS

(Oil bath : 120°C)

Compound (As sulfur 32 mg)	Hydrogen Sulfide (μg) Liberated After 1	3	6 Hours
L-Cystine	21	248	660
L-Cysteine	52	186	450
Djenkolic acid	61	187	338
L-Cysteine mercaptal of acetaldehyde	54	175	315
L-Cysteine mercaptal of *n*-butyraldehyde	41	129	240
L-Cysteine mercaptal of benzaldehyde	75	308	620
L-Cysteine mercaptal of furfural	137.5	450	900
L-Cysteine mercaptole of pyruvic acid	105	274	412.5
L-Cysteine mercaptole of levulinic acid	98	250	403

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 565~570, 1960]

Ciliatine: A New Aminophosphonic Acid Contained in Rumen *Ciliate Protozoa*[*]

Studies on the Reticulo-rumen Digestion. Part XVII.

By Masaaki HORIGUCHI and Makoto KANDATSU

Department of Agricultural Chemistry, the University of Tokyo

Received March 15, 1960

In the course of study on the amino acid composition of sheep rumen Protozoa, a new ninhydrin-positive substance was isolated from acid hydrolysates of a proteolipid-like material of Protozoa. This substance was ideneified as 2-aminoethane phosphonic acid, and the occurrence of a C-P bond in Nature has been demonstrated for the first time.

Aminocarboxylic acids are distributed in all organisms and a lot of new substances of this kind have been discovered in the last decade. Taurine, 2-aminoethane sulfonic acid, is also found to occur in many organisms. However, the occurrence of aminophosphonic acid in nature has not been recognized up to the present. Chavane and Hackspill[1] (1947) suspected that its 'absence' in nature might depend upon its unstability, and proceeded to make investigations into the properties of several synthetic aminophosphonic acids. However, contrary to their expectation, all compounds examined were sufficiently stable so that they might be isolated from organisms.

In the course of study on the amino acid composition of sheep rumen *Ciliate Protozoa*, which probably play an important role in the nitrogen metabolism in reticulo-rumen, an unknown ninhydrin-positive substance was found by paper chromatography (Fig. 1) to be present in acid hydrolysates of the ether-ethanol soluble fraction of Protozoa. The movement of this substance by paper electrophoresis and its behaviors toward the cation- and amion-exchange resins suggested that the substance was a slightly acidic amphoteric ion. This substance was isolated in the crystalline form (Fig. 2) by means of ion-exchange chromatography.

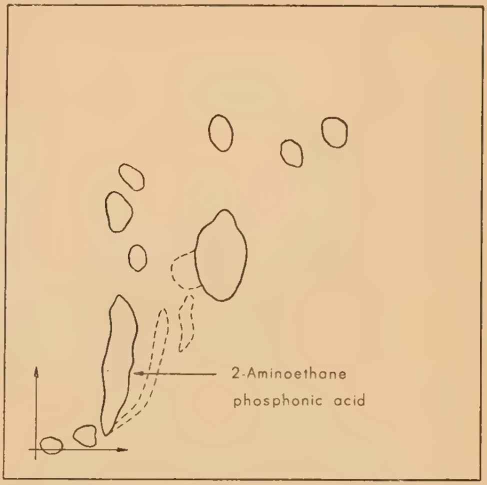

FIG. 1. Paper Chromatograms of Hydrolysed Ether-ethanol Soluble Material from Rumen *Ciliate Protozoa*.

Solvents: phenol: water (4:1) vertical; *n*-butanol: acetic acid: water (4:1:2) holizontal.

The substance melted at 296~299°C with decomposition, and was readily soluble in water,

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[*] Presented at the 193rd Local Meeting of the Agricultural Chemical Society of Japan held in Tokyo, June 27, 1959. A preliminary report of this paper was presented in *Nature*, **184**, 901 (1959).

1) V. Chavane and L. Hackspill, *Compt. rend.*, **224**, 406 (1947).

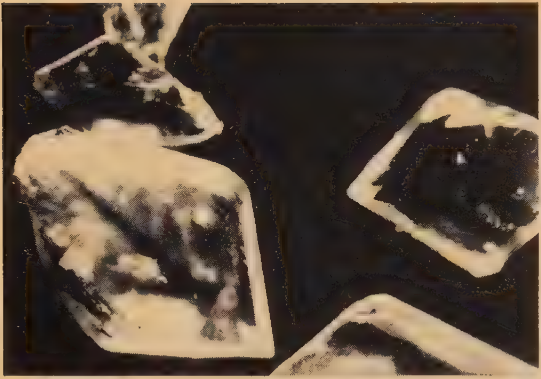

FIG. 2. Crystals from Rumen *Ciliate Protozoa*. (×100)

scarcely soluble in methanol, and insoluble in ethanol, acetone, ether, phenol and glacial acetic acid.

It gave negative tests for halogen and sulfur, and dissolved in hot sodium hydroxide solution without any liberation of ammonia, and the nitrogen was present entirely as amino-N, as shown by the Van Slyke amino nitrogen method.

From the results of elementary analysis, the empirical formula corresponded to $C_2H_8NPO_3$. When the ninhydrin color yield on molar basis relative to leucine was determined, the yield from 296 g of the substance was equivalent to that from 1 mole of leucine (Table I). Provided that this was a monoamino acid ($C_2H_8NPO_3$, Molecular Weight 125) or a diamino acid (Molecular Weight 250), the color yield corresponded to 0.42 or 0.84, respectively. In general, color yields from diamino acids are higher than 1, and those from α-monoamino acids and β-monoamino acids are equal to about 1 and 0.5, respectively. Therefore, it seemed likely that the substance was not a diamino acid, but a monoamino acid. Moreover, as the substance was optically inactive, it was suggested that the amino radical is not attached to the α-carbon. The molecular weight determined by the titration method with alkali (Fig. 3) was 125. Thus, it was presumed that the substance was β-monoamino acid and the molecular formula corresponded to $C_2H_8NPO_3$.

TABLE I. COLOR YIELD FROM 2-AMINOETHANE PHOSPHONIC ACID ON MOLAR BASIS RELATIVE TO LEUCINE

Determined on 2 ml solutions in the buffer at pH 5.0; ninhydrin reagent 1 ml; heating time 15 minutes; diluent 5 ml ethanol-water (1:1); read at 570 mμ.

Compound	Sample amt.	Optical density	Sample amt. required optical density 1		Color Yield
				average	
	μ moles		μ moles	μ moles	
Leucine	0.2822 0.1881 0.1411	0.886 0.590 0.428	0.319 0.319 0.330	0.323	1
	μ g		μ g	μ g	
Isolated substance	66.6 22.2	0.712 0.228	93.5 97.4	95.5	0.42* 0.84**

* Calculated as molecular weight 125
** Calculated as molecular weight 250

The substance decolorized neither potassium permanganate solution nor bromine water. The infrared spectrum determined with 'Nujol mull' (Fig. 4) showed absorption bands at 1658 and $1563\,cm^{-1}$ due to NH_3^+, bands at 1171, 1149 and $1044\,cm^{-1}$ which could be assigned to P=O, but did not show absorption near $1600\,cm^{-1}$ due to COO^-. Upon hydrolysis with 5 N sodium hydroxide in a sealed tube at 120°C for eight hours, no phosphoric acid was released, and so it was suggested that this substance was neither a phosphoric acid ester nor its amide, but phosphorus was directly combined with the carbon atom.

From these results, this substance was presumed to be 2-aminoethane phosphonic acid, which had been synthesized by Finkelstein[2], Kosolapoff[3], Chavane and Hackspill[1], and Chavane[4]. This was synthesized by the authors, and identity of isolated and synthetic compounds was confirmed in accordance with infrared spectra (Fig. 4), R_F values in paper chromatography in 3 solvent systems, and behaviors on ion-exchange columns. Thus, the isolated substance was identified as 2-aminoethane phosphonic acid,

$$H_2N \cdot CH_2 \cdot CH_2 \cdot PO(OH)_2 .$$

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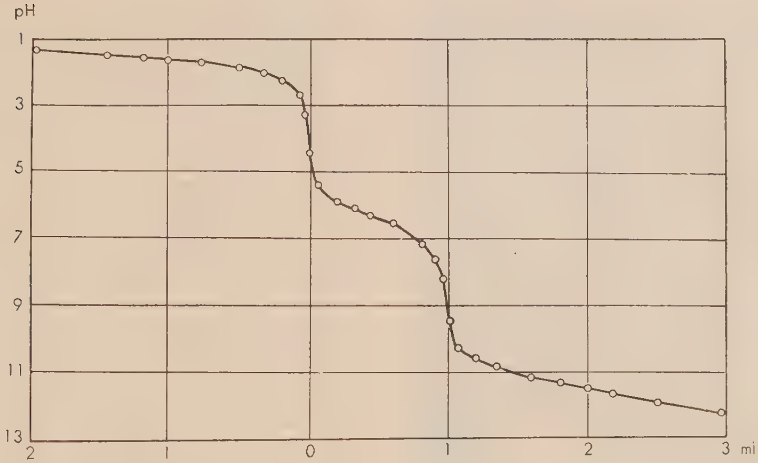
[2] J. Finkelstein, *J. Amer. Chem. Soc.*, **68**, 2397 (1946).
[3] G.M. Kosolapoff, *J. Amer. Chem. Soc.*, **69**, 2112 (1947).
[4] V. Chavane, *Ann. chim.*, [12] **4**, 372 (1949).



N/10 HCl ←—|—→ N/10 KOH

FIG. 3. Titration Curve of 2-Aminoethane Phosphonic Acid.

Determined with 1 ml aqueous solution containing 13.0 mg of the isolated substance.

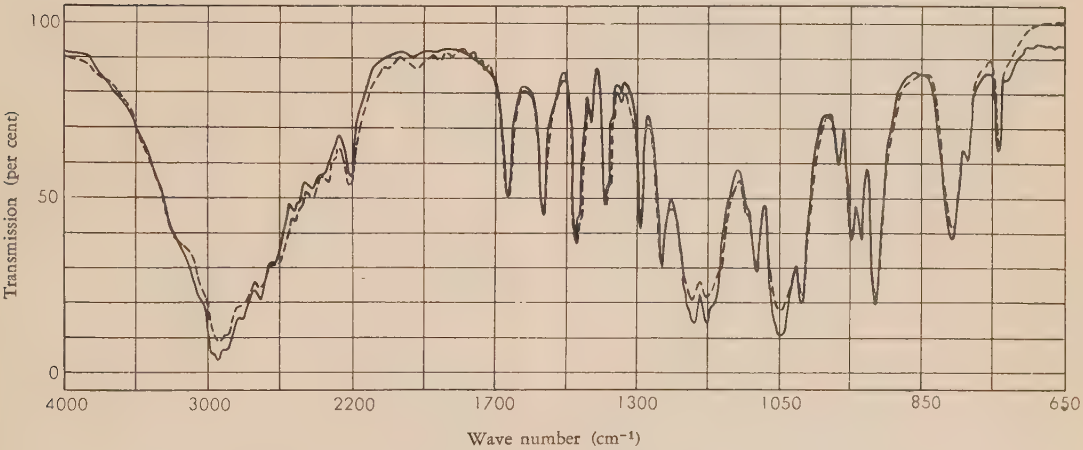


FIG. 4. Infrared Spectra of Isolated and Synthetic 2-Aminoethane Phosphonic Acid. (Nujol mull)

----: Isolated ——: Synthesized

It is proposed to designate this substance by the ideomatic name "Ciliatine".

Phosphorus compounds in organisms, as well as in their products, have been known as orthophosphoric acid, pyrophosphoric acid, metaphosphoric acid, phosphoric acid ester, pyrophosphoric acid ester and phosphoric acid amide, namely, phosphorus is combined with other elements giving O-P, O=P and N-P; now the occurrence of the C-P bond has been demonstrated.

Recently, an insecticide having a C-P bond in its molecule, O, O-dimethyl-2, 2, 2-trichloro-1-hydroxyethylphosphonate, has been imported into Japan, but its use is still restricted within experiment farm limits. It was therefore a most unlikely assumption that rumen Protozoa might have synthesized 2-aminoethane phosphonic acid from this insecticide adhered to the dietary grass fed to experimental sheep. It will be interesting to investigate whether this substance is widely distributed in organisms or occurs only in rumen Protozoa.

The possible use of this aminophosphonic acid as a reference substance of protozoal bodies, its distribution in organisms, mechanisms of biogenesis and physiological functions are now under study.

EXPERIMENTAL

Preparation of Protozoa

Protozoa were obtained by a modification of the procedure of McNaught et al[5]. Rumen contents were taken out through the rumen fistulas of two sheep (Corridale) maintained on daily rations consisting of 250 g of concentrate mixture (equal parts of soybean cake and wheat bran) and 4 kg of green grass or 1 kg of dried grass. The contents were diluted with an equal volume of 0.5% glucose-0.5% sodium chloride solution (39°C), and squeezed through 8-folded surgical gauze. The liquid was then poured into cylinders and allowed to stand in a warm water bath (39°C). After a short time, the floating fibrous layer was removed by suction and the liquid was decanted, and the well-defined layer of larger Protozoa at the bottom was collected. The fibrous layer was well mixed with the glucose-sodium chloride solution and allowed to settle once more to take out any Protozoa trapped in the fibrous materials. The liquid obtained by decantation was centrifuged at 3,000 r.p.m. for one minute and the smaller Protozoa were collected. The combined protozoal fractions were resuspended in the glucose-sodium chloride solution and then treated in a hand operated centrifuge repeatedly until the supernatant layer became quite clear. The latter treatment was essential to obtain Protozoa almost free from bacteria. The Protozoa, which were still actively moving, were then preserved in acetone. After a few days, Protozoa were filtered, washed with acetone until the color of the filtrate disappeared, dried at 105°C and allowed to stand in the air. In all, 50 kg of rumen contents were treated in this way giving a total yield of 203 g of dried Protozoa.

Purity of Protozoal Preparation

Microscopic examination of the protozoal preparation showed that it contained no extracellular materials, and its fiber content measured by the method of A.O.A.C. (1950) was 0.0%.

Indication of Protozoal Types

The fauna consisted of Diplodinium (mainly *D. magii* and *D. ecaudatum*), Isotricha, Ophryoscolex, Dasytricha and Entodinium (mainly *E. simplex*, *E. longinucleatum* and *E. caudatum*). The latter three genera constituted a large portion of the whole.

Isolation of 2-Aminoethane Phosphonic Acid

Two hundred and three grams of dried Protozoa were extracted for ten times with 10 l of hot ether-ethanol (1 : 1). The extract was concentrated to about 50 ml, and allowed to stand in the cold. The resultant sediments were collected by filtration and extracted with 30 ml of ethanol. Upon cooling this ethanol solution, about 6 g of a proteolipid-like material was obtained, and this material was hydrolysed by refluxing for thirteen hours with 100 ml of constant boiling hydrochloric acid. After extracting the resultant lipids with ether, the hydrolysate was taken to dryness and transferred with 4 ml of water to a bed (1.3×4 cm) of 'Dowex 50-X4' (hydrogen form). Then the bed was washed with a small amount of water. The effluent fractions and washings were discarded, and 5% pyridine was percolated through the bed to elute acidic and neutral amino acids. The eluate was taken to dryness and transferred with 2 ml of 0.6 N hydrochloric acid to a column (1.1×58 cm) of 'Dowex 50-X4' (200 mesh, hydrogen form) which was previously equilibrated with 0.6 N hydrochloric acid. Elution was carried out with 0.6 N hydrochloric acid, and the effluent was collected in 5-ml fractions at the rate of 5 ml per hour. Under these conditions, the substance was found in tubes 30~34 separated from other ninhydrin positive substances. Detection was carried out by one-dimensional paper chromatography with *n*-butanol : acetic acid : water (4 : 1 : 2) as a solvent. The pooled effluent fractions containing the substance were concentrated to dryness, and the resultant liquid was transferred with 1 to 2 ml of 0.5 N acetic acid to a bed (1.1×1.4 cm) of 'Dowex 1-X8' (acetate form) to remove the traces of hydrochloric acid. The bed was washed with 0.5 N acetic acid, and the first 7 ml of the effluent usually contained all of this substance. The effluent was concentrated to dryness, dissolved in a small amount of

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[5] M.L. McNaught, E.C. Owen, K.M. Henry and S.K. Kon, *Biochem. J.*, **56**, 151 (1954).

5% acetic acid and treated with a small bed (0.5×1 cm) of decolorizing carbon. Then upon evaporation of acetic acid, the substance was obtained in crystalline form and was recrystallized twice from water-ethanol, dried in Abderhalden drying apparatus at 110°C under 5 mm Hg for three hours in a yield of 63 mg of tiny rhombic crystals. The substance was slightly hygroscopic and melted at 296～299°C (uncorr.) with decomposition.

Elementary Analysis

Phosphorus was analysed by Lohmann and Jendrassik's modification[6] of the Fiske and Subbarow method[7] following the wet ashing process with sulfuric acid.

Found: C, 19.57; H, 5.66; N, 11.34; P, 24.7%.
Calcd. for $C_2H_8NPO_3$: C, 19.20; H, 6.55; N, 11.20; P, 24.8%.

Determination of Amino Nitrogen

The Van Slyke amino nitaogen method was employed. Nitrogen was entirely released within five minutes reaction time at room temperature (21°C).

Amino-N from 3.219 mg of sample: Found, 366μg; Calcd., 361μg.

Measurement of Molecular Weight

Owing to the lack of a convenient solvent for the substance, Rast's method could not be employed, and the molecular weight was estimated as described above by the following ninhydrin color yield method and titration method.

Determination of Ninhydrin Color Yield

Color yield from the isolated substance on molar basis relative to leucine was determined by the method of Moore and Stein[8] (Table I). The absorption maximum of the substance was at about 570 mμ the same as that of amino-carboxylic acids.

Titration Curve

To obtain a titration curve (Fig. 3), 1-ml solutions, each containing 13.0 mg of the substance, were titrated with 1/10 N potassium hydroxide and 1/10 N hydrochloric acid, and pH values were successively determined by a Glass Electrode pH Meter. The PKa value obtained was 6.4.

Molecular weight, determined by alkali titration: 134.
Molecular weight, calculated for $C_2H_8NPO_3$: 125.

Measurement of Optical Rotation

Optical rotation was measured with aqueous solution containing 12.5 mg of the substance per 1 ml in a 10-cm cell at 15°C. The observed value (−0.02°) was within the range of experimental error (±0.03°).

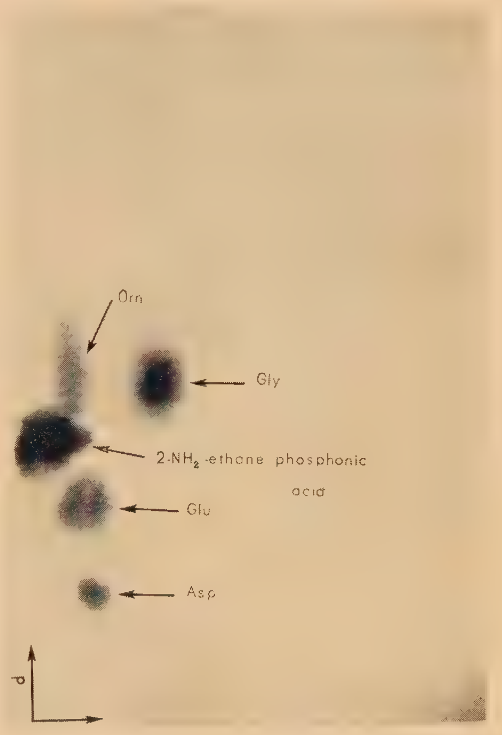

FIG. 5. Map of Location of 2-Aminoethane Phosphonic Acid on Chromatograms.

Solvents: phenol: water (4:1) vertical; 2,4-lutidine: aniline (9:1) saturated with water horizontal.
Orn: ornitine; Gly: glycine; Glu: glutamic acid; Asp: aspartic acid.

Infrared Absorption Spectra

Infrared spectra (Fig. 4) were determined with 'Nujol mull', and showed absorption bands at 2203, 1658, 1563, 1289, 1230, 1171, 1149, 1078, 1044, 1018, 971, 953, 934, 914, 807, 782 and 739 cm^{-1}.

Synthesis of 2-Aminoethane Phosphonic Acid

The procedure of Chavane[4] was employed except that traces of hydrochloric acid were removed by a bed of 'Dowex 1-X8' (acetate form) prior to crystallization with water-ethanol. This substance melted at 296～298°C with decomposition; Found: N, 11.23%. Infrared spectrum of this substance in 'Nujol mull' is shown in Fig. 4.

Paper Chromatography

Paper chromatography by the ascending technique was carried out on Toyo No. 51 Filter Paper with (I) *n*-butanol: acetic acid: water (4:1:2), (II) phenol: water (4:1), and (III) 2,4-lutidine: aniline (9:1) saturated with water. The isolated and synthetic compounds behaved identically on paper chromatograms with these three solvent systems.

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[6] K. Lohmann u. L. Jendrassik, *Biochem. Z.*, **178**, 418 (1926).
[7] C.H. Fiske and Y. Subbarow., *J. Biol. Chem.*, **66**, 375 (1925).
[8] S. Moore and W.H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

The location of 2-aminoethane phosphonic acid on chromatograms run in phenol : water (4 : 1) and 2,4-lutidine : aniline (9 : 1) saturated with water is shown in Fig. 5. R_F values were as follows :

Solvent (I) : 0.14 ($\fallingdotseq$ Lysine)
Solvent (II) : 0.31 ($\leqq$ Serine, $>$Glutamic acid)
Solvent (III) : 0.02 ($<$Ornitine)

Acknowledgement The authors wish to express their sincere thanks to Mr. K. Aizawa for infrared spectra measurements and also to the members of the Microanalytical Laboratories of this Department.

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Measurements of Catalatic Reaction by an Electronic Manometer*

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Received March 15, 1960

Although the manometric study of the catalatic reaction has several drawbacks in comparison with the titrimetric method, the manometry has often been used to determine the catalatic activity of an impure preparation because the oxygen evolution from hydrogen peroxide is owing exclusively to the action of catalase.

To overcome the drawbacks a sensitive recording electronic manometer was constructed using a RCA 5734 subminiature triode, a mechano-electronic transducer, with diaphragms, which was reliable for the studies of not only the catalatic reaction but also other reactions which accompany a rapid or minute pressure variation. Measurements of the catalatic reaction with the apparatus was satisfactorily compared with the titrimetry.

INTRODUCTION

It has already been established by titrimetric[1)] and spectrophotometric[2)] studies that the catalatic rate is satisfactorily described by first order kinetics except for special cases, for example, in the presence of organic acids in an acidic medium[3)]. However, as already pointed out by Beer et al.[4)], manometric studies have not given data accurate enough for a kinetical study. This shortcoming of the manometry is mainly due to the slow equilibration of oxygen[5),6)] between gas and liquid phase in the reaction vessel. Vigorous stirring of the reaction mixture to overcome this unfavorable situation causes a denaturation and inactivation of the catalase protein. Because of these reasons, it is said that a high accuracy of the measurement can not be expected in the manometry.

Although the manometry has these inherent drawbacks, it has often been used because of an advantage that the enzyme preparation for the assay with this method need not be purified. The oxygen evolution is exclusively due to the specific action of catalase. For example, the catalatic activity of native mitochondria preparation[7] is successfully measured by the manometry without mitigating the integrity of the particles. Furthermore, in practice, it is simple, rapid and makes it possible to follow the reaction continually.

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* This report was presented for the Meeting of the Agricultural Chemical Society of Japan at University of Tokyo, April 1959.

1) R.K. Bonnichsen, B. Chance and H. Theorell, *Acta Chem. Scand.*, **1**, 685 (1947).
2) R.F. Beer, Jr. and I.W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).
3) R.F. Beer, Jr., *J. Phys. Chem.*, **59**, 552 (1955).
4) R.F. Beer, Jr. and I.W. Sizer, *Science*, **117**, 710 (1953).
5) P. George, *Biochem. J.*, **43**, 287 (1948).
6) F.J.W. Roughton, *J. Biol. Chem.*, **141**, 129 (1941).

Up to the present 80 or more manometric measurements have been done for catalase[8]. Almost all of these were not suited for the estimation of the catalatic activity in view of the fact that the manometry resulted in only 3 to 30% of the rate constants obtained by other methods. By using a strain gauge as the manometer sensing element which needs a pressure difference of 40 to 80 mm Hg, Greenfield et al.[9] have recovered a high activity, although their manometer is not extremely sensitive.

The authors report a design of a sensitive electronic manometer and experimental results with it which is satisfactory for the kinetical studies of not only the catalatic reaction but also other rapid reactions which accompany a gas evolution or consumption.

EXPERIMENTALS

Enzyme preparation. A crystalline preparation was obtained by the method of Kitagawa and Shirakawa[10] from cow liver, which had a specific activity of $2.7\times 10^{7}(\mathrm{M}^{-1}\cdot\mathrm{sec.}^{-1})$ at 0°. The calculation of the specific activity was based on the estimation of haematin content by the method of Keilin and Hartree[11], and the measurement of first order rate constant at 0° by the rapid permanganate titrimetry[1]. Prior to the manometric studies, the enzyme activity was determined titrimetrically. This preparation had a specific activity of 3.6×10^{7} $(\mathrm{M}^{-1}\cdot\mathrm{sec.}^{-1})$ at 20°.

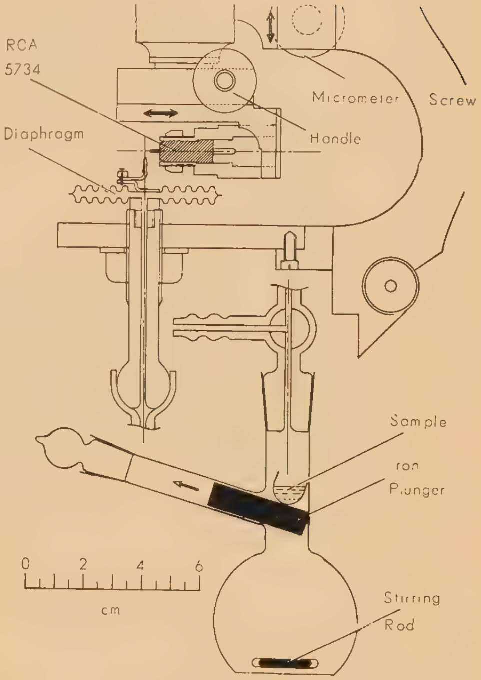

FIG. 1. Sectional Diagram of the Transducer Assembly and Reaction Vessel.

Apparatus. The construction of the apparatus, which has proved to be reliable, is shown in Fig. 1, with a complete view of it in Fig. 2. For the sensing element a RCA subminiature triode was adapted with a pressure diaphragm. A brass block bored to a diameter just allowing the valve to be inserted and to be held firmly without undue strain, was attached to a micrometer head *via* a horizontal displacement mechanism which consisted of a rack and pinion system. The movement of the pressure diaphragm, connected to the reaction vessel, is transmitted to the plate shaft which extends through a thin metal diaphragm on the top of the 5734, through a knife edge soldered to the center of the pressure diaphragm. By adjusting the distance of the contact point of the knife edge from the plate shaft of the 5734, it is possible to change the pressure range for the measurements, and also to start with any pressure. The reaction vessel was connected to the sensing assembly by means of a standard semi-ball joint.

The reaction vessel, 90 ml in capacity, had a side arm in which an iron plunger was inserted. The vessel, dipped into a water bath controlled to 20±0.10°, was

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7) R.E. Greenfield and V.E. Price, *J. Biol. Chem.*, **220**, 607 (1956).

8) A.C. Mahley, in David Glick, "Methods of Biochemical Analysis", Interscience Publishers, New York, 1954, **Vol. 1**, p. 357.

9) R.E. Greenfield and V.E. Price, *J. Biol. Chem.*, **209**, 355 (1954).

10) M. Kitagawa and S. Shirakawa, *J. Agri. Chem. Soc. Japan*, **18**, 308 (1942).

11) D. Keilin and E.F. Hartree, *Biochem. J.*, **49**, 88 (1951).

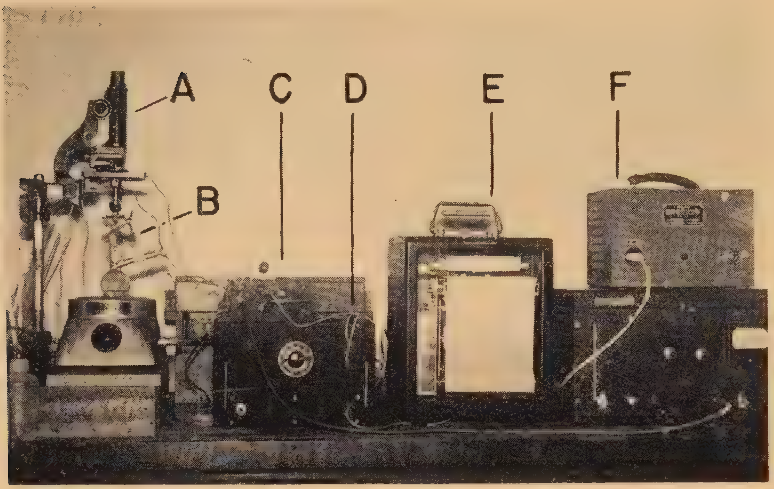

FIG. 2. Photograph of the Electronic Manometer in Position.
A, transducer; B, reaction vessel; C, bridge; D, amplifier; E, pen-recorder; F, electric source and AC voltage stabilizer. Water bath is omitted to show the reaction vessel.

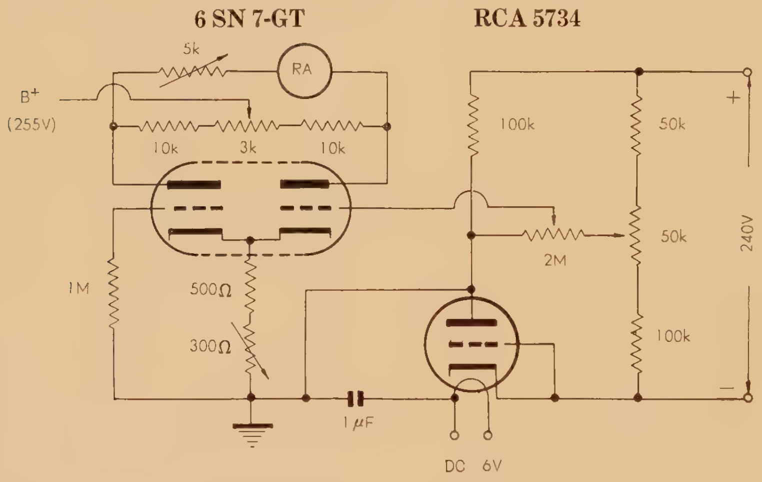

FIG. 3. Circuit Diagram of the Detector and Amplifier.

stirred magnetically from the bottom.

The pressure variation caused in the course of a reaction, results in a vertical micro displacement of the pressure diaphragm, which bend the plate shaft of the 5734. The displacement of the plate shaft causes a change in the distance between the fixed cathode and the plate[12]. This results in a large change in the impedance of the valve. Under no circumstances, the plate shaft was subjected to displacement from its normal position by more than 0.5 degree.

The main electronic circuit diagram is shown in Fig. 3. The RCA 5734 triode was connected in a bridge circuit which was first balanced by adjusting the variable resistor of 50 kΩ**. The attenuator of 2 MΩ was included for the convenience of balancing the bridge circuit and to provide a proper input for a single-stage pen-recorder driving unit. The unequilibriated bridge current was recorded immediately after the start of the reaction. The chart speed of the recorder*** was set

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12) H.F. Olson, *J. Acoust. Soc. Amer.*, **19**, 307 (1947).

** A potentiometer type of an accurate degree.

*** Full scale: 2 mA, internal resistance 1.8 kΩ. Yokogawa Electric Works, Inc. Type KR-1.

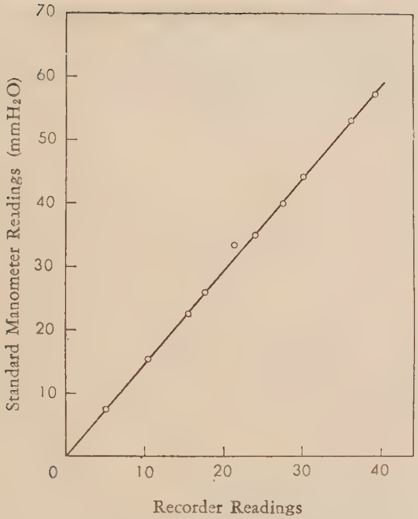

FIG. 4. Sensitivity of the Instrument and Relation between Pressure and Recorder Readings.
Full scale of the recorder readings is 40 (cf. Fig. 5).

to 120 or 60 mm/min. in any cases.

Procedure. Ten ml of a buffered solution of the substrate was placed in the reaction vessel and 0.1 ml of enzyme solution pipetted in a cup was put on the iron plunger. The conditions of the manometry were made identical with those used for the titrimetry, 0.01 N hydrogen peroxide as substrate and 0.04 M phosphate buffer, pH 6.75, for the convenience of comparison.

After the equilibriation of the electronic circuit, temperature and gas distribution in the reaction vessel, the plate shaft was allowed to barely touched the knife edge, giving a small deflection of the recorder pen. And then the bridge circuit was again equilibriated. The catalatic reaction was started by pulling the plunger into the side arm to allow the cup to fall into the substrate mixture by means of a magnet.

Calculation. The recorder readings was calibrated against a standard manometer, connected to the apparatus through a three-way stop cock shown in Fig. 1, by adjusting the amplifier gain. The relation of standard manometer and the recorder readings, which shows the sensitivity of the manometer, is presented in Fig. 4. In the present experiments, the lever ratio of the plate shaft (touching point of knife edge to plate shaft) was kept constant, so a unit of the recorder readings, which was proportional to 0.3μ of the diaphragm movement, corresponded to 1.5 mm H$_2$O.

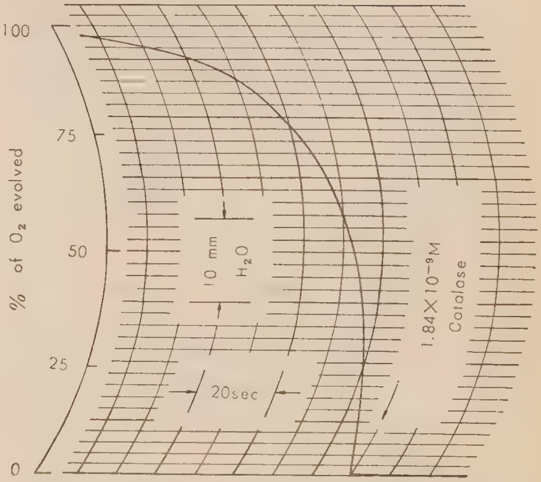

FIG. 5. A Recording of Oxygen Evolution Rate.
Experimental condition was 0.01 N hydrogen peroxide in 0.04 M phosphate buffer, pH 6.75, at 20°.

The linearity was consistent up to 100 mm H$_2$O.

The amount of oxygen to be evolved from the substrate was quickly checked by using a large amount of catalase before one series of measurements. The first order rate constant of the catalysis was computed from the slope of the linear portion of semilogarithmic plots of residual oxygen against the reaction time.

RESULTS AND DISCUSSION

Reliability of the measurements. Fig. 5 shows an example of a recording. Fig. 6 is semilogarithmic plots of oxygen evolution rate based on such recordings. A time lag was recognized during the initial phase of the reaction for 5 to 10 sec., but afterward a linearity was obtained until 80 to 90% completion of the reaction. The plots of the rate of the hydrogen peroxide decomposition followed by titrimetry under the same condition as in the manometry showed a parallelism to the linear portions of the manometric data as indicated in Fig. 6. This phenomenon should be due to the action of steady state membrane of oxygen diffusion which forms at interfaces as already mentioned[6,13,14]. The time lag can not be attributable to a in-

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13) W.K. Lewis and W.G. Whitman, *Ind. Eng. Chem.*, **16**, 1215 (1924).

14) H.S. Davis and G.S. Crandall, *J. Am. Chem. Soc.*, **52**, 3757 (1930).

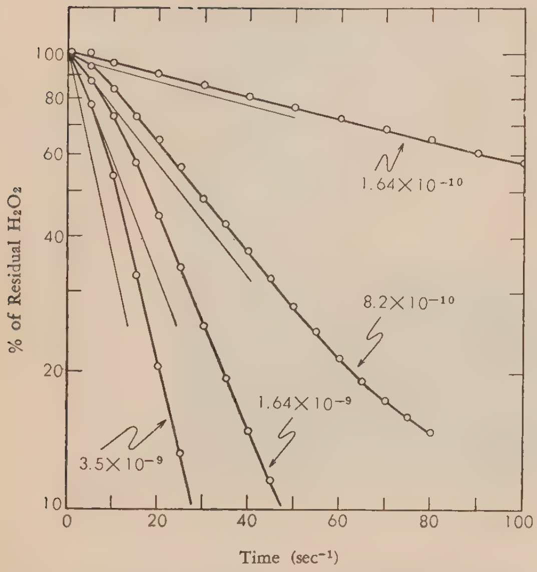

FIG. 6. Semilogarithmic Plots of Catalatic Reaction.

Thin lines indicate the titrimetric data. Figures represent the concentration of catalase. The amounts of residual hydrogen peroxide were read off from the recordings at every 5 or 10 sec. after the start of the reaction.

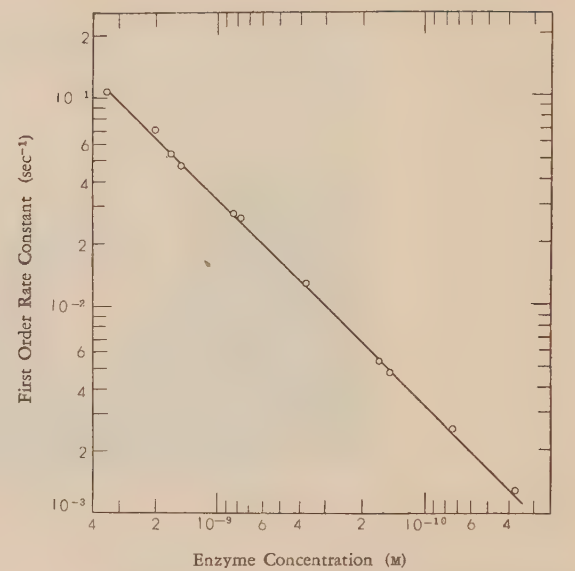

FIG. 7. Proportionality of the Rate Constant and the Concentration of Catalase.

strumental problem, as the apparatus has a high frequency response[15]. By using a pure enzyme preparation a progressive inactivation of catalase was observed, although in the case of rather impure enzyme preparations this phenomenon was not detectable.

The logarithmic plots of the first order rate constant calculated from the linear portion against the enzyme concentration gave a straight line having the slope of 45 degree exactly as shown in Fig. 7. There is a linear proportionality between enzyme concentration and the rate constant obtained from the manometry, when the enzyme concentration was between 3×10^{-9} and 4×10^{-10} M. Above this limit, oxygen gas diffusion rate from the liquid to gas phase seemed to become constant irrespective of enzyme concentration and below this limit a blank evolution of oxygen possibly due to a vigorous stirring was not negligible.

Catalatic activity under reduced pressure. No difference was found between the experimental data obtained under reduced and normal pressures (100 and 760 mm Hg). These results at least supported that oxygen diffusion rate from the catalytic site of catalase to the bulk solution is not a rate controlling factor in the over-all catalatic reaction. This conclusion is also supported by the experiments, in which the gas phase of the reaction vessel was replaced by oxygen gas, did not show any difference in the rate from the ordinary condition.

The authors have obtained data which indicate that the diffusion of the substrate into the catalytic site is a rate controlling factor in over-all kinetics of the catalatic reaction, which will be reported separately****.

Stability of the apparatus. When the instrument has been switched on the thirty min. or so the electrical drift is extremely small. Replacement of the 5734 has not yet been necessary in the apparatus which has been used frequently for nearly two years.

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15) M. Ainworth and J.W. Eveleigh, *J. Sci. Instr.*, **31**, 471 (1954).

**** An outline of it was presented for the Symposium on the Enzyme Chemistry, Japan, at Kyoto University, July 1959.

Acknowledgement The authors wish to express sincere thanks to Prof. H. S. Mason, Department of Biochemistry, University of Oregon Medical School, Oregon, U.S.A., for kindly providing a RCA 5734 for our use. This study was supported by the Grant in Aid for Agricultural Research from the Ministry of Education.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 575~586, 1960]

Studies on Lactic Acid Fermentation

Part I. Triose Metabolism*

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Received March 16, 1960

Resting cells of *L. fermentum* convert glyceraldehyde to equimolar lactic acid and neither the evolution of carbon dioxide nor the uptake of oxygen was observed. Glyceraldehyde-3-phosphate and 3-phosphoglycerate were identified as intermediates which were equally labeled with inorganic P^{32} in reaction systems, and the presence of triokinase was suggested.

DeMoss and his collabolators[1,2] have recently reported the studies on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from *Leuconostoc mesenteroides*, whereas Horecker and his coworkers[3,4] have demonstrated the presence of phosphoketolase in the extract of *Lactobacillus pentosus*.

It would be reasonable to presume that with general heterofermentative microorganisms glucose, even in anaerobic fermentation, seems to undergo some initial oxidative cleavages to produce pentose phosphate, followed by the formation of glyceraldehyde-3-phosphate by phosphoketolase, and that no decarboxylation is included, to produced two-carbon fragment in the matabolic process from triose phosphate to lactic acid with these bacteria. In this paper, the authors report that *Lactobacillus fermentum* is able to consume triose and also describe the studies on its metabolic steps as well as on its products with the detection of some significant intermediates.

MATERIALS AND METHODS

Cell growth. Cells of *L. fermentum* and *Leuc. mesenteaoides* grown on 10 l of malt extract (5.5° Brix), containing 0.1% yeast extract, at 37°C for two days, were harvested with Sharples centrifuge, washed twice with 100 ml of distilled water, and then suspended in 0.05 M KCl. After three hours for starvation the washed cells were resuspended in the same solution to obtain 50 mg cells of dry weight per ml, and used for overall experiments as cell suspension.

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[1] R.D. DeMoss, I.C. Gunsalus and R.C. Bard, *J. Bacteriol.*, **66**, 10 (1953).

[2] R.D. DeMoss and M. Gibbs, *J. Bacteriol.*, **70**, 730 (1955).

[3] B.L. Horecker et al., International Synposium on Enzyme Chemistry (1957).

[4] J. Hurwitz, *Biochim. et Biophys. Acta*, **28**, 599 (1958).

* Presented at the General Meeting of the Agricultural Chemical Society of Japan, Kyoto, May 8, 1958.

Enzyme preparation. Crude cell-free extracts were prepared by sonic oscillation at 10 kc for 30 minutes followed by centrifugation in the cold at 7,000 rpm for 20 minutes. To the clear supernatant 3 times volume of 0.1 M tris buffer (pH 7.8) was added and dialyzed overnight against 150 times volume of 0.05 M KCl.

Chemicals. Glyceraldehyde-3-phosphate was prepared by the method of Meyerhof and Junovicz-Kocholaty[5], 3-phosphoglycerate by the method of Neuberg and Lustig[6], and DPN, ATP and DL-glyceraldehyde were commercial materials. Glyceraldehyde-3-phosphate dehydrogenase was prepared by the procedure of Krebs[7] from baker's yeast, which either possessed negligible activity or failed to reduce DPN in the presence of glyceraldehyde, ethanol or α-glycerophosphate.

Analytical. Lactic acid was determined by the method of Barker and Summerson[8] and volatile acid by the modification of the method of Kirk[9,10]. Ethanol was determined by the oxidation with permanganate. Triose, triose phosphate and phosphoglycerate were eluted from paperchromatogram and estimated by the methods of Lohmann and Meyerhof[11], Dische[12] and Rappoport[13], respectively. In addition, glyceraldehyde-3-phosphate dehydrogenase was used for the identification of triose phosphate which was detected in reaction mixtures. Pyruvate was determined by the procedure of Friedeman and Haugen[14], both inorganic and organic phosphates by the method of Long and King.[15] acetoin and diacetyl by Westerfeld[16] and White et al.[17] and protein by the method of Lowry[18]. Optical measurements were carried out with a Beckman DU-model Shimazu spectrophotometer, and radioactive phosphate was counted with Geiger-Müller counter.

RESULTS

The results shown in Table I indicate that intact cell suspension of *L. fermentum* possesses apparently far stronger activity of methylene blue reduction in Thunberg tube when triose was used as a starting substrate than of *Leuc. mesenteroides*, and that the velocity of the former was nearly one-third of the glucose. These figures show mean values of duplicate tubes in each case.

When the experiments in Table I were carried out with tris buffer instend of phosphate, no evidence demonstrated direct dehydrogenation of triose. If triose was incubated statically with cell suspension of *L. fermentum* in a 50 ml-Erlenmeyer flask for about two hours, confirmation of equimolar formation of lactic acid from triose was observed (Table II); the data in Expt. 1 show that none of other expected

TABLE I. METHYLENE BLUE REDUCTION BY CELL SUSPENSION OF *L. fermentum* AND *L. mesenteroides*

Bacteria	Substrate	Time for decolorization (min.)
L. fermentum	glucose	8
〃	glyceraldehyde	22
〃	—	>180
L. mesenteroides	glucose	6
〃	glyceraldehyde	120
〃	—	>180

Each Thunberg tube constained: 2 ml of 0.1 M phosphate buffer (pH 7.2), 40 μmoles of $MgCl_2$, 0.3 μmoles of methylene blue, 3.3 mg of resting cells in suspension, 25 μmoles of substrate, in a total volume of 4 ml. Temperature, 37°C; interior atmosphere, 20 mmHg.

TABLE II. PRODUCTION OF LACTATE FROM TRIOSE

Inhibitor	Substrate consumed	Products		
		Lactate	Ethanol Acetate	Acetoin Diacetyl
	μmol	μmol	μmol	μmol
—	8.75	8.19	0	0
2,4-Dinitrophenol	5.32	4.91	0	0
iodoacetate	1.23	0.52	0	0
—	—	0.08	0	0

Reaction mixture contained: 0.1 M phosphate buffer (pH 7.5), 10 ml; $MgCl_2$, 2.4 mg; cells in suspension, 168 mg; glyceraldehyde, 10 mg; 2,4-dinitrophenol, 5×10^{-4} M; iodoacetate, 2×10^{-4} M; in a total volume of 40 ml. The mixture was statically incubated for 120 minutes at 37°C.

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5) O. Meyerhof and Junovcz-Kocholaty, *J. Biol. Chem.*, **149**, 71 (1943).

6) C. Neuberg and H. Lustig, *Arch. Biochem. and Biophys.*, **1**, 311 (1942).

7) E.G. Krebs, *Methods in Enzymol.*, **Vol. I**, 407 (1955).

8) D.B. Barker and W.H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).

9) P.L. Kirk, *Anal. Chem.*, **22**, 611 (1950).

10) K. Kimura and S. Fukudo, *J. Agr. Chem. Soc. (Japan)*, **27**, 516 (1953).

11) K. Lohmann and O. Meyerhof, *Biochem. Z.*, **273**, 60 (1934).

12) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1949).

13) D. Rappoport, *Biochem. Z.*, **291**, 429 (1937).

14) T.E. Friedemann and G.E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

15) C. Long and E.J. King, *Biochem. J.*, **26**, 296 (1932).

16) W.W. Westerfeld, *J. Biol. Chem.*, **161**, 495 (1945).

17) A.G.C. White et al., *Arch. Biochem. and Biophys.*, **9**, 229 (1946).

18) O.H. Lowry, et al., *J. Biol. Chem.*, **193**, 265 (1951).

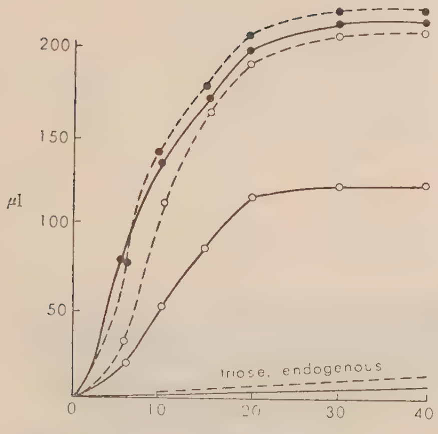

FIG. 1. Lack of Gas Metabolism with Triose by Living Cell Suspension of *L. fermentum*

Each Warburg cup contained: 0.05 M phosphate buffer (pH 6.2), 0.8 ml; $MgCl_2$, 10 μmoles; substrate, 10 μmoles; resting cells, 5.8 mg; in a final volume of 2.3 ml. Gas phase, air; temperature, 37°C. Real curves represents evolution of CO_2, and dotted curves uptake of O_2.

●: glucose ○: gluconate

products such as acetate, acetoin and diacetyl were detected besides trace of pyruvate which is supposed to be an intermediate.

In these experiments, iodoacetate at low concentration (Expt. 3) shows remarkable inhibition not only on the consumption of triose but also on the production of lactate, while with 2, 4-dinitrophenol (Expt. 2), though 40 to 50% inhibition was observed, no effect on molar equilibrium between substrate utilization and lactate formation were observed. Furthermore, the following investigation indicates that the conversion of triose to lactate is accompanied neither by production of carbon dioxide nor by uptake of oxygen (Fig. 1); after forty minutes' incubation the disappeared substrate and the products in each cuvette were analyzed, and the results gave evidence for lack of both oxygen necessity and carbon dioxide evolution during the triose dissimilation because approximately equimolar lactate had yielded against to consumed triose. It is suggested that no degradation to furnish C_1- and C_2-fragments is contained in the metabolic route.

TABLE III. INFLUENCE OF METHYLENE BLUE ON GLUCOSE, GLUCONATE AND TRIOSE UTILIZATION BY *L. fermentum*

Expt. No.		Substrate utilized	Methylene blue added	Products		
				Lactate	Ethanol	Acetate
		mmol	μmol	μmol	μmol	μmol
1	glucose	1.0	10	939	877	117
2	//	1.0	1,000	804	413	516
3	//	1.0	2,000	756	24	1,023
4	gluconate	1.0	10	873	457	588
5	//	1.0	1,000	812	58	1,015
6	glyceraldehyde	0.5	500	374	0	1,015

Reaction mixture contained: phosphate buffer (pH 7.5), 2.5 mmoles; tris buffer (pH 7.8), 400 μmoles; substrate, 1.0 mmoles; $MgCl_2$, 50 μmoles; $MnSO_4$, 15 μmoles; methylene blue, as indicated; resting cells, 800 mg; in a total volume of 75 ml. Semi-anaerobic incubation was carried out for three hours at 37°C. The dye was added dropwise to the stirred mixture.

A mode of lactic acid fermentation has been reported by Katagiri and Imai[19], where the presence of hexose monophosphate pathway was suggested with a series of experiments on aerobic fermentation of gluconate. For the reaffirmation of the above experiments from a different site, the following procedure was adopted where the cell suspension of *L. fermentum* utilizes the same substrate (glucose and gluconate) or triose by the addition of equimolar or duple-molar methylene blue, which was semi-anaerobically added dropwise (Table III). The data obtained provides a good confirmation to the previous assumption; two couples of electron stepwise liberated from glucose molecule (or a couple of electron from gluconate) were accepted with methylene blue and the substrates undergo an aerobic cleavage to produce equimolar lactate, acetate and carbon dioxide as if air oxygen were vigorously supplied. In Table III, Expts. 1 and 4 show no significant effect on the mode of the substrate dissimilation with a small amount of the redox dye, while if equimolar dye to mole of glucose is presented the ethanol production decreases as if normal fermentation of gluconate which proceeded anaerobically (Expt. 2); approximately equimolar ethanol and

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[19] H. Katagiri and K. Imai, *J. Agr. Chem. Soc.* (*Japan*), **19**, 15[illegible] (1955).

TABLE IV. TRIOSE CONVERSION BY CRUDE EXTRACTS OF *L. fermentum*

Expt. No.	Inhibitor	Substrate utilized	Products			
			G-3-P*	3-PGA**	Lactate	Pyruvate
		μmol	μmol	μmol	μmol	μmol
1	—	27	0.8	25	17	trace
2	iodoacetate	5.8	3.2	0	6.2	—
3	dinitrophenol	15	0.4	1.7	11	—
4	〃	23	0.7	1.9	18	—
5	〃	29	—	0	23	—
6	—	—	—	0	0.6	—

Reaction mixture contained: phosphate buffer, 250 μmoles (pH 7.5); $MgCl_2$, 20 μmoles; ATP, 5 μmoles; glyceraldehyde, 40 μmoles; iodoacetate, 2×10^{-4} M; 2,4-dinitrophenol, 5×10^{-4} M; extract, 1.25 ml; in a final volume of 8 ml. The system was incubated statically at 30°C for 60 minutes. In Expt. 4, 10 μmoles of ATP was added. In Expt. 5, triose phosphate was used as substrate instead of glyceraldehyde. In expt. 6, no substrate was added.

* Glyceraldehyde-3-phosphate, ** 3-phosphoglycerate

acetate were detected in a final broth. In the case of triose (Expt. 6), no significant inhibition on the production of lactic acid was observed possibly because the rodox system participating in the production of lactic acid is different from that for the formation of ethanol.

Similar inhibition was observed in the case of crude cell-free extracts (Table IV); with the extracts no less appreciable decrease of lactic acid formation took place by the addition of either iodoacetate or dinitorphenol compared with cell suspension. The former was found to be a powerful inhibitor on triose consumption, more than 60% inhibition occurring at 2.0×10^{-4} M, while with the latter, in the presence of more amount of ATP, inhibiting ability on substrate conversion seems to be considerably extinguished (Table IV, Expt. 5), following the accumulation of triose phosphate. From the above fact, it is possible to consider that SH-group of triose phosphate dehydrogenase appears to be attacked with iodoacetate even in its low concentration. On the contrary, dinitrophenol could not diminish utilization of glyceraldehyde (Expt. 6).

Therefore the possibility is suggested that triose is first phosphorylated to give glyceraldehyde-3-phosphate, prior to dehydrogenation by triose phosphate dehydrogenase. It may be also elucidated that inhibiting phenomenon by trace amount of dinitrophenol in these results is due to the difficulty of preliminary phosphorylation of triose by decrease of ATP concentration which is obviously resulted from inhibition on electron-oxygen-transfer (e-o-t) level in the crude extracts.

TABLE V. PAPERCHROMATOGRAPHY OF METABOLIC INTERMEDIATES

Substance	R_F value with solvent system of		
	I	II	III
Authentic samples;			
orthophosphate	0.31	0.68	0.42
G-3-P	0.40	0.52	0.45
3-PGA	0.35	0.62	0.38
Tested compounds;			
A	0.39	0.50	0.45
B	0.34	0.60	0.39

I. isopropanol: isobutanol: conc. NH_4OH: water (40:20:1:39) [1*]
II. isopropanol: trichloroacetic acid: conc. NH_4OH: water (75:5g:0.3:25) [1*]
III. butyric acid:0.5N-NH_4OH (pH 3.6) (10:6) [2*]
1* J. P. Ebel, *Bull. Soc. Biol. Chem.*, **34**, 330 (1952),
2* B. Magasanik et al., *J. Biol. Chem.*, **186**, 51 (1950).

For the identification of intermediates each reaction mixture after two hours' incubation deproteinized supernatant treated as described by Umbreit[20] was chromatographed on filter paper. Hanes-Isherwood[21] reagent was used for the detection of the phosphorylated compounds, and the spots were eluted with distilled water to be available for specific reactions.

Spot A in Table V showed apparent reducing power due to its carbonyl radical; it was posi-

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[20] W.W. Umbreit et al., Manometric Techniques and Tissue Metabolism, 185 (1951).

[21] C.S. Hanes and F.A. Isherwood, *Nature*, **164**, 1107 (1949).

TABLE VI. INCORPORATION OF P^{32} INTO PHOSPHORYLATED COMPOUNDS BY THE EXTRACT OF *L. fermentum*

Inhibitor	P^{32}added	Incorporated products		
		G-3-P^{32}	3-P^{32}GA	ATP^{32}
	cpm/μmol	cpm/μmol	cpm/μmol	cpm/μmol
—	2580	450	522	872
iodoacetate	2580	385	410	720
dinitrophenol	2580	74	315	36

The reaction conditions were the same as those described in Table IV, except that 4.5 μmoles of DPN, 10 μmoles of ATP and dialyzed enzyme were used.

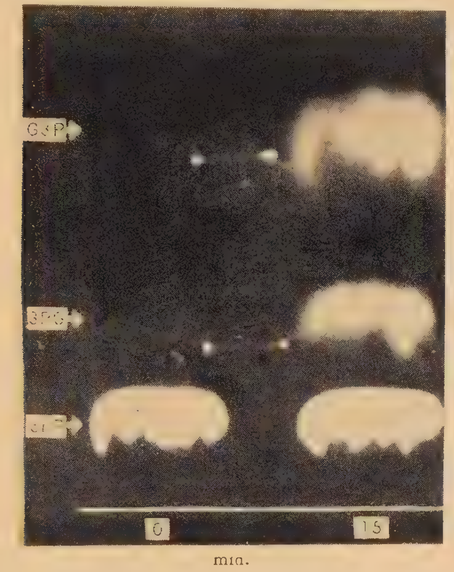

FIG. 2. Radioautogram of P^{32}-labeled Intermediates in Triose Conversion by the Extracts of *L. fermentum*.

Fuji X ray film was kept radiated for 3 days on the paper-chromatogram, which was developed by double ascending method with the following solvents. First solvent, *n*-propanol: conc. NH_4OH: water (60:30:10). Second solvent, phenol: water (82:18).

tive to aniline hydrogen phthalate and blue with phenylenediamine. The substance gave a strong absorption band at 650 mμ with cysteine-carbazole and at 600 mμ with orcinol reagent. When the substance was kept in 1 N NaOH for fifteen minutes at 100°C, approximately theoretical amount of inorganic phosphate was liberated. It was also observed that in the presence of glyceraldehyde-3-phosphate dehydrogenase, the material tested could reduce DPN spectrophotometrically nearly as much rapidly as authentic glyceraldehyde-3-phosphate.

Spot B gave deep yellow tint depending on its carboxyl radical by bromphenol blue spray reagent, and its eluate brought forth pink color by resorcinol corresponding to the absorption at 500 mμ, and gave blue color by naphthoresorcinol which was absorbed at 670 mμ. No destruction of ester linkage of the phosphate in question was observed by heating at 100°C for thirty minutes in 1 N HCl. These several tests described above identified spot A as glyceraldehyde-3-phosphate and spot B as 3-phosphoglycerate.

In order to investigate the utilization of inorganic phosphate for the phosphorylated intermediates in the enzymatic system of *L. fermentum*, radioactive orthophosphate (P^{32}) was mixed to non-radioactive phosphate buffer (Table VI); after the incubation period, the reaction mixtures were treated as described in the experimental section, and radioautographed for two days. The results in Figure 2 show clear incorporation of inorganic P^{32} into either phosphorylated intermediates.

DISCUSSION

Several results presented in this report suggest that conversion of triose by *L. fermentum* may coincide with the pathway which is widely found in yeast as well as homolactic acid fermenters; triose phosphate is stepwise converted to lactic acid via classical Embden-Meyerhof scheme. Every enzyme and enzyme system in *L. fermentum*, however, still remain unresearched. It is possible to assume that behavior and specificity of the enzyme group in this organism have similarity to those in yeast. As one of the most interesting findings in the present report, indirect evidence for the presence of a triokinase in this organism, which have been previously reported by Hers[22] in liver, awaits further investigation.

It may not be essential to postulate that phosphorylation of triose to its phosphate ester in heterolactic microorganisms is dependent on an inducible system formed in response to contact with triose, because, even though knowledge of the mechanism of triose utilization is still fragmentary, no lag period is required for its consumption either with the living cells or with the extracts.

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[22] J.G. Hers, *Biochim. et Biophys. Acta*, **11**, 423 (1953).

That dihydroxyacetone phosphate could not be detected in each reaction mixture appears to indicate that triose phosphate isomerase may be exceedingly inferior or inactive in the extracts of *L. fermentum.* Moreover, the results on methylene blue reduction presented in Table I seem to indicate the dehydrogenation of glyceraldehyde-3-phosphate which is subsequently formed by preliminary phosphorylation of triose.

Although no evidence for the complete absence of a triose dehydrogenase which catalyzes only direct dehydrogenation of glyceraldehyde might be led from the present data, observation on stoichiometric experiments favors certainty of triose conversion to lactate via well-known Embden-Meyerhof pathway in great part.

SUMMARY

1. *L. fermentum* can utilize glyceraldehyde, forming almost equimolar lactic acid per triose consumed. Neither the evolution of carbon dioxide nor the uptake of oxygen was observed manometrically, and any by-product such as volatile acid, ethanol or acetoin was not detected in the process of its dissimilation.

2. Glyceraldehyde-3-phosphate, 3-phosphoglycerate and pyruvate were identified as intermediates of triose metabolism ; the two formers were equally labeled with inorganic P^{32} in reaction systems.

3. Considerable degree of inhibition by both iodoacetate and dinitrophenol on triose disappearance as well as on lactic acid formation was observed. In consequence, possibility of the presence of Embden-Meyerhof route for triose utilization and some relating problems are discussed.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 581~587, 1960]

Studies on Lactic Acid Fermentation

Part II. Mannitol Utilization*

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Resting cells of lactic acid bacteria oxidize mannitol only aerobically to produce lactic acid, accompanied by theoretical oxygen uptake. The presence of mannitol dehydrogenase in the extracts of heterofermenters as well as mannitokinase and mannitol-1-phosphate dehydrogenase in the extracts of homofermenters were demonstrated. DPN-dependence of both dehydrogenases and the reversibility of the reactions were ascertained. Mannitol dehydrogenase was partially purified from *L. brevis*, which is specific for mannitol and activated by cobalt ion.

Mannitol has been known as one of the three naturally occurring hexitols (sorbitol, dulcitol and mannitol). Peterson and coworkers[1,2] studied mannitol production from fructose by *Lactobacillus pentoaceticus*. Mannitol fermentation by members of lactobacilli has been reported by Katagiri and Kitahara[3] and Kitahara[4], in which homofermentative microorganisms can utilize mannitol, whereas heterolactic fermenters will not be practically able to ferment mannitol though these organisms anaerobically produce large amount of mannitol from fructose.

In the present report, difference of mode of the utilization of mannitol between homo- and hetero-fermentative microorganisms is indicated, and mannitol dehydrogenase was partially purified from the extracts of *Lactobacillus brevis*.

EXPERIMENTAL

Organism and enzyme preparation: All microorganisms were grown on malt extract containing 0.1 % dried yeast extract for two days at 37°C. The cells were harvested by a single passage through a Sharples continuous supercentrifuge at a rate of about 300 ml per minute and washed twice with water by centrifugation. The washed cells were suspended in 0.1 M tris buffer (pH 7.8) at a final concentration of 80 mg dry weight per ml, and disrupted by treating for forty minutes in a Raytheon sonic disintegrator (9 Kc/sec.). The extract was clarified by centrifuging of 11,000 rpm at 0°C for fifty minutes and dialyzed overnight against 0.1 M tris buffer (pH 7.8) at 4°C and then the clear dialyzate obtained was stored in deep freeze before use.

Analytical: Mannitol was determined by oxidation with periodate[5]. Reducing sugars were estimated by the method of Folin and Malmros[6]. Fructose and F-6-P were identified by the methods of Roe[7] and Dische[8]. All spectrophotometric measurements were made with a Shimazu spectrophotometer. Other analytical methods were the same as those described in the previous paper[9].

Chemicals: Fructose-6-phosphate was prepared by hydrolysis of fructose-1,6-diphosphate with HCl. Mannitol-1-phosphate was kindly supplied by Dr. S. Tanaka, Department of Biochemistry, Faculty of Science, Kyoto University. Xylitol was prepared from xylose by the reduction with borohydride[10]. For paperchromatography Toyo filter paper (No. 51) was used.

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* Presented at the General Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 5, 1959.

1) W.H. Peterson and D.B. Fred, *J. Biol. Chem.*, **41**, 431 (1920).

2) W.H. Peterson and E.B. Fred, *ibid.*, **42**, 273 (1920).

3) H. Katagiri and K. Kitahara, *Nippon-Gakujutsu-Kyokai-Hokoku*, **16**, 548 (1942).

4) K. Kitahara, *J. Agr. Chem. Soc. (Japan)*, **16** 819 (1940).,

5) C.D. West and S. Rappaport, *Proc. Soc. Exp. Biol. and Med.*, **70**, 141 (1949).

6) O. Folin and J. Malmros, *J. Biol. Chem.*, **83**, 115 (1929).

7) J.H. Roe, *J. Biol. Chem.*, **107**, 15 (1934).

8) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 538 (1951).

9) H. Katagiri, K. Imai and K. Ito, This Bulletin, **24**, 575 (1960).

10) J.B. Wolf and N.O. Kaplan, *J. Biol. Chem.*, **218**, 852 (1956).

RESULTS

1. Requirement of Oxygen for Mannitol Utilization.

As a preliminary experiment, ability of ferment mannitol as well as to produce lactic acid from the polyol was tested both aerobically and anaerobically. Data in Table I show that both species of microorganisms used were capable of utilizing mannitol aerobically, and that in a semi-anaerobic (static) condition, in which the reaction mixture was incubated statically in a 100 ml-Erlenmeyer flask with a cotton plug, no volatile acid was formed from mannitol, whereas in aerobic condition, where a shaking incubator was used, production of acetic acid was prominently increased in proportion to the decrease of lactic acid or ethanol formation. These results have good agreement with those reported previously by Katagiri[3]. The accordance of the superficial results described above suggests that mannitol should be metabolized as the end products through the metabolic pathway containing the well-known sugars or their phosphate esters.

In Figs. 1 and 2, some evidence is presented manometrically for the presumption that the most plausible shunt of mannitol utilization would be one that originates from initial oxidation of the substrate to fructose or frutose-6-phosphate.

In aerobic utilization of mannitol (Fig. 1)

TABLE I. STOICHIOMETRY OF MANNITOL FERMENTATION

Organism	Mannitol utilized	Products		
		Lactate	Ethanol	Acetate
	μmol	μmol	μmol	μmol
L. casei:				
aerobic	758	1,040	0	243
semi-anaerobic	159	294	0	0
semi-anaerobic (G)	(824)	1,460	0	151
L. brevis:				
aerobic	1,000	790	20	1,170
semi-anaerobic	359	275	330	0
semi-anaerobic (G)	(920)	858	680	144

Reaction mixture contained: phosphate buffer (pH 7.0), 2.5 μmoles; mannitol, 1 mmole; $MgCl_2$, 4.5 μmoles; $MnSO_4$, 20 μmoles; resting cells, 250 mg; $CaCO_3$, 300 mg in a total volume of 50 ml. (G) in semi-anaerobic conditions shows that equimolar glucose was added as substrate instead of mannitol. In aerobic condition the reaction mixture was shaken in a 500 ml-shaking flask at 30°C for 2 hours, and in semi-anaerobic condition, static incubation was carried out at 30°C in a 100 ml-Erlenmeyer flask for 5 hours.

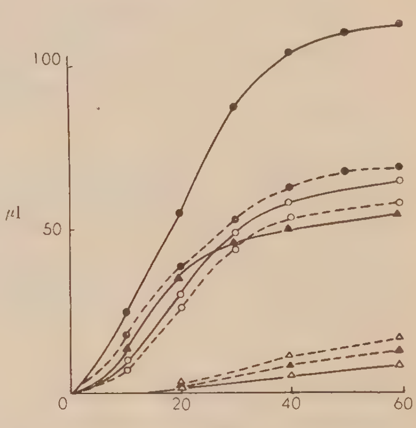

FIG. 1. Aerobic Utilization of Mannitol.

Each Warburg cup contained: mannitol (or fructose), 3.5 μmoles; phosphate buffer (pH 6.0), 100 μmoles; $MgCl_2$, 15 μmoles; resting cells, 5.5 mg in a total uolume of 2.3 ml. Gas phase, air; temperature, 37°C. After 70 minutes, 0.1 ml of 10 N H_2SO_4 was added, then an aliquot of the mixture was pippeted and assayed for lactic acid. Real curves represent gas metabolism of mannitol, and dotted curves that of fructose.

Heterofermenters: *L. brevis, L. fermenti, L. mesenteroides*

O_2 (●——●, ●- - -●), CO_2 (○——○, ○- - -○)

Homofermenters: *L. casei, L. plantarum*

O_2 (▲——▲, ▲- - -▲), CO_2 (△——△, △- - -△)

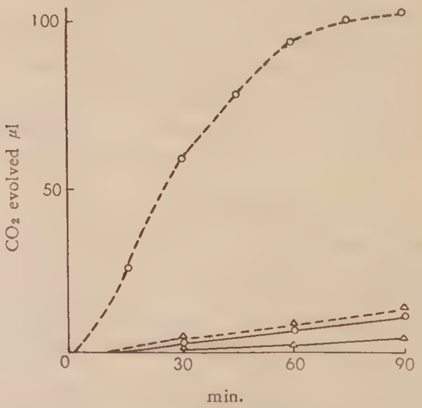

FIG. 2. Anaerobic Utilization of Mannitol.

Experimental conditions were the same as described in Fig. 1, except that the interior gas phase was substituted for nitrogen gas and that after 90 minutes the contents were analyzed. The symbols used represent the same as described in Fig. 1.

TABLE II. FORMATION OF REDUCING SUGARS BY CRUDE EXTRACTS

Organism	O_2-uptake with		Reducing sugars formed from	
	Mannitol	Mannitol-1-phosphate	Mannitol	Mannitol-1-phosphate
	μmol	μmol	μmol	μmol
L. casei	0.2	2.8	0	4.9
L. plantarum	0.1	3.7	0	5.8
L. fermenti	6.4	0	12.5	0
L. brevis	9.0	0	15.8	0
Leuc. mesenteroides	8.2	trace	14.7	trace

Reaction mixtures contained: 120 μmoles of phosphate buffer (pH 6.0), 15 μmoles of $MgCl_2$, 20 μmoles of substrate and 0.2 ml of extracts (dialyzed overnight) in a final volume of 2.3 ml. The mixture was incubated aerobically for 90 minutes at 37°C.

either homofermenter or heterofermenter took up oxygen vividly, the amount of which was almost correspondent to one half of moles of consumed substrate. Nevertheless, theoretical amount of lactic acid was detected in each reaction mixture. From these phenomena it would be assumed that one mole of reduced coenzyme (DPNH) resulted from the first dehydrogenation of mannitol combines via electron transfer system, with one half mole of air oxygen aerobically without any coupling transport of electron in vivo.

On the contrary, in absolutely anaerobic circumstances, even though fructose as well as glucose was readily consumed and gave good yield of lactic acid in the same condition (Fig. 2), mannitol was hardly fermented by both organisms. This indicates that these organisms cannot utilize mannitol in anaerobic condition; supply of air oxygen was indispensable to the initial degradation of mannitol by these microorganisms.

According to the appearance of oxygen consumption in a manometer, it would be more likely that mannitol undergoes first deprivation of two hydrogen atoms by these organisms. The crude extracts of either *L. casei* or *L. plantarum*, however, utilize practically little amount of mannitol when the extracts were dialyzed overnight against 0.05 M trios buffer, and yet the dialyzed extracts are still active on mannitol-1-phosphate instead. In the case of heterolactic fermenters, the appearance was just otherwise: their extracts, when dialyzed overnight in the same manner, showed no detectable loss of activity to attack mannitol, while even non-dialyzed extracts of these microorganisms are obviously inactive on mannitol-1-phosphate from the outset (Table II, Fig. 3). From these data described above, it would be manifested that reaction system of mannitol utilization in both members of lactobacilli can be formulated as the following equations:

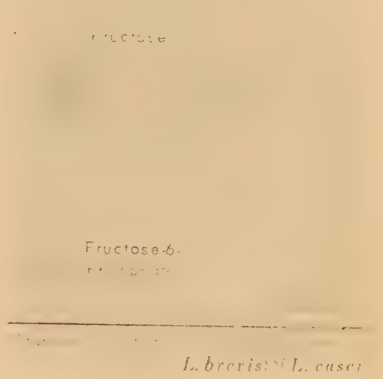

FIG. 3. Paperchromotogram of the Products in Mannitol Oxidation by Hetero- and Homofermentative Microorganisms.

The paper was developed with the solvent system (phenol: water, 80:20) for 30 hours at room temperature. After drying, the sugars were spotted out by spraying of aniline hydrogen phthalate solution.

Homolactic species;

$$\text{Mannitol} + \text{ATP} \longrightarrow \text{Mannitol-1-phosphate} + \text{ADP} \quad (1)$$

$$\text{Mannitol-1-phosphate} + \text{DPN}^+ \rightleftharpoons \text{Fructose-6-phosphate} + \text{DPNH} + \text{H}^+ \quad (2)$$

Heterolactic species;

$$\text{Mannitol} + \text{DPN}^+ \rightleftharpoons \text{Fructose} + \text{DPNH} + \text{H}^+ \quad (3)$$

Evidence for the presence of mannitol dehydrogenase in heterofermenters and mannitol-1-phosphate dehydrogenase in homofermenters

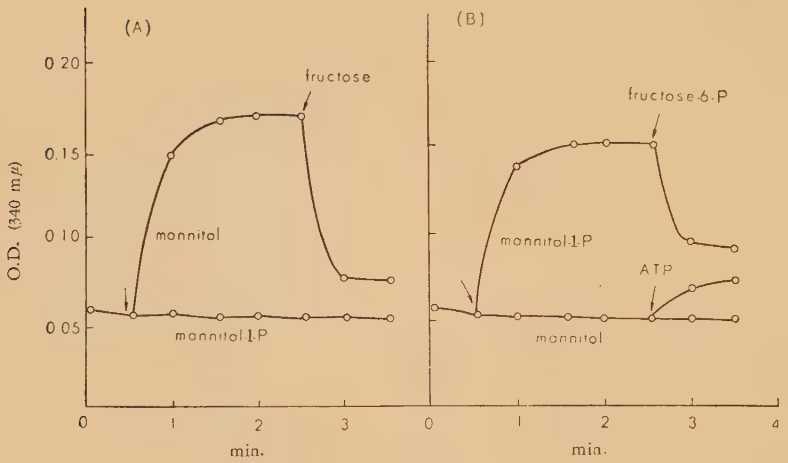

FIG. 4.

(A) Reversibility of mannitol oxidation with mannitol dehydrogenase. Reaction mixture contained: tris buffer (pH 7.8), 150 μmoles; cell-free extracts, 0.01 ml; DPN, 0.15 μmole in a total volume of 3.8 ml. Subsequent additions were 20 μmoles of mannitol, 15 μmoles of mannitol-1-phosphate or 40 μmoles of fructose in order, as indicated with allows. Change of optical density was read before and after addition of the substrate in 30 second-intervals.

(B) Reversibility of mannitol-1-phosphate oxidation with mannitol-1-phosphate dehydrogenase. Experimental conditions were the same as described in (A), except that subsequent addition was 35 μmoles of fructose-6-phosphate or 1 μmoles of ATP.

was clearly established with the spectrophotometric procedure (Fig. 4). These figures show that cell-free extracts of *L. brivis* grown on malt extract is able to reduce DPN in the presence of mannitol, but not of mannitol-1-phosphate and thereafter reduced DPN is rapidly reoxidized reversibly with subsequent addition of excess of fructose. Hence it would be demonstrable that the reaction of mannitol dehydrogenase from these organisms is easily, at least in vitro, reversible and that the enzyme seems absolutely dependent on DPN, since it cannot reduce TPN in the presence of mannitol.

With respect to homolactobacilli, it is of interest to explain that in their extracts no reduction of DPN occurs by the addition of mannitol, but the addition of mannitol-1-phosphate readily results increase of optical density at 340 mμ due to the reduction of DPN. The enzyme also requires DPN as coenzyme, and TPN was impossible to replace DPN. Consequently, these results show that in homolactic fermenters mannitol-1-phosphate dehydrogenase exists, and that mannitokinase (or mannitol-1-phosphatase) seems to participate in the utilization of mannitol since DPN is gradually reduced even with the addition of mannitol if small amount of ATP is added (Reaction (1)).

Analogues of mannitol-1-phosphate dehydrogenase which catalyzes Reaction (2) have been detected and studied in details in *Escherichia coli*. However, mannitol dehydrogenase, which seems to catalyze Reaction (3), has not yet been thoroughly investigated with the groups of lactic acid bacteria. Therefore, it is of interest as well as of significance to study further on the latter enzyme. Partial purification of the enzyme from *L. brevis* was carried out and its properties were investigated.

2. Purification of Mannitol Dehydrogenase from *L. brevis*.

The enzyme which catalyzes Reaction (3) was partically purified from *L. brevis*. All purification proceudres were carried out in the cold as far as possible.

Assay method for activity of the enzyme: Reaction mixture (3.5 ml) contained 200 μ moles of tris buffer (pH 7.8), $CoSO_4$ (3×10^{-4} M), 25 μmoles of mannitol, 0.03 μmole of DPN and the enzyme. Change of optical density at 340 mμ due to the reduction of DPN was read spectrophotometrically at 20-second intervals before and after additions of substrate.

Definition of unit of the enzyme: One unit was defined as the amount of the enzyme to result change 0.01 of optical density per minute in a light path of 1 cm at 30°C throughout all procedures.

Purification of the enzyme:

Step 1. First fractionation with ammonium sulfate.

Crude extract of *L. brevis* was first concentrated by means of ammoniun sulfate fractionation; solid ammonium sulfate was added to the extract with stirring up to 0.30 saturation and the faint precipitate was removed off and discarded. Then the saturation of the supernatant was raised to 0.38 with further addition of solid ammonium sulfate and the turbid solution was centrifuged at 11,000 rpm for thirty minutes. The precipitates gathered were disdissolved in 0.1 M tris buffer (pH 7.8) which usually contained as much activity as 75% of total activity in the initial extracts. For the following operation the solution was dialyzed for five hours against distilled water to remove off large part of cations.

Step 2. Rivanol fractionation.

The dialyzed enzyme solution was next treated with immediate addition of rivanol: 0.25 ml of 0.1% rivanol was dropwise added to the dialyzate (25 ml, 3.7 mg protein per ml) with vigorous stirring and small amount of brownish yellow precipitate was centrifuged off and discarded. To the transparent supernatant, 0.5 ml of the same rivanol solution was further added and the precipitate coagulated was successively washed twice with small volume of distilled water, and thereafter dissolved with 0.5 M phosphate buffer (pH 6.0). After the residue was centrifuged off, the yellow supernatant was decolorized with refreshed acid clay. Colorless protein solution was dialyzed for two hours against running tap water and then against 0.1 M phosphate buffer (pH 7.0) containing 10^{-4} M glutathione overnight, during which several changes of dialyzing water are effective.

Step 3. Ca-phosphate gel adsorption.

To the cold dialyzate, which was adjusted to pH 5.8 with 5 N acetic acid, 13.8 mg of gel was added. After vigorous stirring for a few minutes the adsorbed protein was eluted from the gel first with 5.8 ml of 0.1 M phosphate buffer (pH 6.8), next with the same buffer of pH 7.4, and finally at pH 8.2. In many cases, large part of the activity of mannitol dehydrogenase was found in the latest eluate.

Step 4. Second fractionation with ammonium sulfate.

To the final eluate, saturated ammonium sulfate solution was carefully added and precipitated fraction between 0.37 and 0.45 saturation was gathered by centrifugation. The precipitate was dissolved in 0.1 M tris buffer (pH 7.8) and stored in freeze. Storage for two weeks at −20°C resulted in no loss of activity. Table III shows the summarized results of all procedures for its purification. The final preparation was concentrated approximately 40-fold of the original extract and contained negligible activity for glucose-6-phosphate, 6-phosphogluconate and ethyl alcohol.

Properties of mannitol dehydrogenase: The enzyme purified from the extract of *L. brevis* appears absolutely dependent upon DPN as

TABLE III. SUMMARY OF THE RESULTS OF PURIFICATION

	Total activity units	Specific activity units/mg of protein
Crude extracts	2,700	8.1
Ammonium sulfate fraction	1,480	20.6
Rivanol fraction		
Ca-phosphate gel treatment;	1,310	117
1st eluate	20	
2nd eluate	243	
3rd eluate	930	202
Ammonium sulfate precipitate	711	291

TABLE IV. SUBSTRATE SPECIFICITY OF MANNITOL DEHYDROGENASE

Substrate	Relative activity %
Mannitol	100
Mannitol-1-phosphate	0
Sorbitol	7
Dulcitol	0
Xylitol	0
Glycerol	0
Glycol	0

Reaction mixture contained the same components as those described in the assay method for the enzyme except that 0.10 mg of purified enzyme (rivanol fraction) was added.

described before. Several attempts to link the enzyme preparation with TPN were unsuccessful in the presence of mannitol. Also with respect to substrate specificity of the enzyme, as shown in Table IV, none of the polyols such as dulcitol, xylitol, glycerol or glycol, was active as substrate except sorbitol which seems poorly active, presuming because of the cotamination with trace of mannitol. These reslts elucidate that the enzyme from *L. brevis* is different in substrate specificity from thati n *Acetobactor*¹¹⁾. In addition, mannitol-1-phosphate was also inactive as substrate to the enzyme.

Range of optimal pH appears rather wide and its peak roughly locates near 8.0. Among metallic cofactors cabalt ion is to be the most intense activator for the enzyme reaction. Zn^{++} and Ni^{++} were slight effective activators, while Mg^{++}, Mn^{++} and Fe^{++} were all inactive. The stimulatory effect of sulfhydryl compounds such as glutathione or cysteine varied with the stage of preparation and the age of the enzyme. The enzyme was inhibited by Hg^{++}, Ba^{++} and Pb^{++} at relatively low concentration. That addition of EDTA inhibited the reduction of DPN, suggests the requirement of metallic ion for the enzymatic reaction of mannitol dehydrogenase. Substrate specificity polyol dehydrogenase in some microorganisms will be discussed later.

DISCUSSION

All four species of lactic acid bacteria were able to utilize mannitol, rapidly or slowly, in the presence of air oxygen, whereas these microorganisms could hardly convert mannitol to lactic acid in a strictly anaerobic condition. These data suggest that in lactic acid bacteria, used for the experiments in the present paper, mannitol undergoes preliminary dehydrogenation, giving fructose as a direct oxidation product, and that fructose is subsequently metabolized according to species of microorganism. In a static condition, slow oxidation of mannitol causes the production of small amount of lactic acid, but no appreciable disappearance of the substrate might be expected within a short period of incubation. This observation on the slow utilization of the substrate is in good agreement with that by Peterson et al.

Up to date, only few reports have been presented for the enzymatic studies on mannitol dehydrogenase in lactobacilli. Wolf and Kaplan¹²⁾ have indicated that crude extracts of *Leuc. mesenteroides* have no detectable activity for mannitol, where the strain of this organism would be the same as a mannitol-nonproducing heterofermenter.

The assumption that mannitol dehydrogenase of mannitol-producing heterofermenters such as *L. brevic*, *L. fermenti* and *Leuc. mesenteroides* would be adaptive enzyme, could be ruled out since the activity of the enzyme both in proliferating cells and in crude extracts is constant and independent on the presence or absence of mannitol in growth media.

Sebek and Randles¹³⁾ have reported that Pseudomonas can grow on hexitol adaptively and that sorbitol-grown cells can oxidies mannitol as well as sorbitol, in producing fructose from the former and sorbose from the latter. Polyol dehydrogenase from mammalian liver reversely oxidizes D-sorbitol to fructose and L-iditol to L-sorbose¹⁴⁾. The same enzyme has been found in accessory sexual organs of the rat which

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11) A.C. Arcus and N.L. Edson, *Biochem. J.*, **64**, 385 (1956).

12) J.B. Wolf and N.O. Kaplan, *J. Biol. Chem.*, **218**, 852 (1956).

13) O.K. Sebek and C.I. Randles, *J. Bacteriol.*, **63**, 693 (1952).

14) R.L. Blakley, *Biochem. J.*, **49**, 257 (1951).

oxidizes L-arabitol and ribitol[15].

Some hexitol phosphate dehydrogenases appear to be adaptive since kinases are formed during adaptation of microorganism to an added carbohydrate: formation of adaptive specific kinase for arabinose and ribose was reported by Cohen[16], and adaptive phosphorylation of galactose-1-phosphate in yeast are comparable to appearance of kinases for hexitols[17]. A plausible but not entirely satisfactory explanation of its activity on several polyols is the combination of a stable DPN-linked constitutive mannitol dehydrogenase with an adaptive enzyme of L-iditol dehydrogenase type. D-Mannitol dehydrogenase in lactobacilli has the distinct specificity for substrate as described by the Bertrand-Hadson rule, whereas DPN-linked D-sorbitol dehydrogenase has very recently reported in *L. casei*; the enzyme oxidizes sorbitol to fructose[18]. Utilization of mannitol-1-phosphate and lack of oxidizable activity on mannitol by the extracts of homofermentative microorganisms are comprehensive since appreciable amounts of mannitol-1-phosphate were isolated from *L. arabinosus* by Baddiley[19].

SUMMARY

1. Resting cells of both homo- and heterofermentative lactic acid bacteria oxidized mannitol aerobically to produce lactic acid, where half mole of oxygen per mole of utilized mannitol was found to be consumed monometrically. But anaerobically no consumption of the substrate was observed.

2. The crude cell-free extracts of heterofermenters oxidized mannitol to fructose, accompanied by approximately theoretical yield of reducing sugars, and those of homofermenters oxidized mannitol-1-phosphate to fructose-6-phosphate in the same manner, and not *vice versa.*

3. The presence of mannitol dehydrogenase in the extracts of heterofermenters, and mannitokinase and mannitol-1-phosphate dehydrogenase in the extracts of homofermenters were demonstrated spectrophotometrically. DPN-dependence of both dehydrogenase and reversibility of the reactions were ascertained.

4. Mannitol dehydrogenase was purified and about 40-fold active preparation was obtained from *Lactobacillus brevis*. The enzyme is specific for mannitol and activated by cobalt ion.

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[15] H.G. William-Ashman and J. Banks, *Arch. Biochem. and Biophys.*, **50**, 513 (1954).

[16] S.S. Cohen, *J. Biol. Chem.*, **189**, 617 (1951).

[17] R.E. Trucco et al., *Arch. Biochem. and Biophys.*, **18**, 137 (1948).

[18] T.E. Shockley and H.S. Pride, *J. Bact.*, **77**, 695 (1959).

[19] J. Baddiley and A.P. Mathias, *J. Chem. Soc. (London)* **1945**, 2723.

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Studies on Lactic Acid Fermentation

Part III. Mechanism of Ethanol Formation*

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Pyruvate and acetaldehyde were detected as intermediates in the pathway of heterolactic fermentation in the presence of semicarbazide by resting cells of *L. fermenti*. Alcohol dehydrogenase in ammonium sulfate fraction catalyzes both DPN- and TPN- linked dehydrogenation of ethanol to acetaldehyde, and acetyl coenzyme A was reduced to acetaldehyde in the presence of either TPN or DPN. By coupling of xylose degradation with mannitol utilization or by the continuous supply of reduced methyl viologen, appreciable increase of ethyl alcohol was observed.

Acetaldehyde dehydrogenase was reported with *Clostridium kluyveri*[1], and with *Escherichia coli*[2], which is absolutely dependent on DPN and coenzyme A (CoA). It is possible to assume that in heterolactic fermentation acetyl phosphate (or acetyl coenzyme A) produced from xylulose-5-phosphate by phosphoketolase can be reduced up to ethanol when four hydrogen atoms are supplied as if ethanol were produced from glucose.

In the present paper, intermediary formation of acetaldehyde and interconversion of acetyl phosphates, acetaldehyde and ethanol by the extract of *Lactobacillus fermenti* are investigated.

MATERIALS AND METHODS

Preparation of enzyme solution: *L. fermenti* was grown and harvested as previously described[3]. Cells were disintegrated with sonic oscillator for thirty minutes and centrifuged with Spinco centrifuge at 21,000 rpm for thirty minutes. Glutathione (2×10^{-4}M) was added to the supernatant then solid ammonium sulfate was added to 25～80% saturation, and the resulted precipitate was centrifuged. The gathered fraction was dissolved in tris buffer (0.05 M, pH 7.8) and dialyzed overnight against 150 volumes of the same buffer containing 2.0×10^{-4}M glutathione. The enzyme solution was then treated with 0.2 volume of Dowex-1 resin for twenty minutes at 2°C and stored in deep freeze.

Analytical: Methyl viologen chloride was synthesized by the procedure of Smith[4]. Platinum asbestos (30%) was commercial material. Acetaldehyde was measured by the method of Barker and Summerson[5]. Coenzyme A was prepared by the procedure of Stadtman and Kornberg[6], acetyl phosphate and acetyl CoA were synthesized by the methods of Avison[7] and Simon and Shemin[8], and were estimated by the method of Kaplan and Lipmann[9]. Other analytical methods were the same as described in the previous paper[3].

RESULTS

Suspension of resting cells of *L. fermenti* which are harvested from malt extract showed vigorous consumption of glucose to give lactic

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[1] R.M. Burton and E.R. Stadtman, *J. Biol. Chem.*, **202**, 873 (1953).

[2] E.A. Dawes and S.M. Foster, *Biochim. et Biophys. Acta*, **22**, 252 (1956).

[3] H. Katagiri, K. Imai and K. Ito, This Bulletin, **24**, 575 (1960),

[4] C.R. Smith, *J. Am. Chem. Soc.*, **48**, 414 (1924).

[5] D.B. Barker and W.H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).

[6] E.R. Stadtman and A. Kornberg, *J. Biol. Chem.*, **203**, 47 (1953).

[7] A.W.D. Avison, *J. Chem. Soc.* (*London*), **1955**, 732.

[8] E. Simon and J.D. Shemin, *J. Am. Chem. Soc.*, **75**, 2520 (1953).

[9] N.O. Kaplan and F. Lipman, *J. Biol. Chem.*, **164**, 37 (1948),

TABLE I. INTERMEDIARY PRODUCTION OF PYRUVATE AND ACETALDEHYDE FROM GLUCOSE

Expt. No.	Glucose utilized	Products (μmol)				
		Lactate	Ethanol	Acetate	Pyruvate	Acetaldehyde
	μmol					
1	1,000	762	874	120	163	50
2	1,000	922	943	88	2	0
3	—	23	0	0	0	0

Reaction mixture contained: glucose, 1,000 μmoles; semicarbazide, 1,000 μmoles; $MgCl_2$, 50 μmoles; tris buffer (pH 7.8), 3.0 mmoles; phosphate buffer (pH 7.8), 3.0 mmoles; resting cells of *L. fermenti*, 340 mg in a final volume of 50 ml. The mixture was incubated statically at 37°C. Expt. 1, complete system; Expt. 2, without semicarbazide; Expt. 3, without substrate.

acid, ethanol, carbon dioxide and small amounts of acetic acid (Table I). If excess of semicarbazide was added to the reaction mixture from the beginning of glucose utilization, intermediary production of pyruvate as well as acetaldehyde occurred due to the formation of each semicarbazones, which were identified with the authentic samples by the paperchromatogram, by melting point, or by elemental analysis [Found: C, 40.20; H, 2.96; N, 20.0. Calcd. for $C_9H_8N_4O_6$ (pyruvic 2, 4-dinitrophenyl hydrazone): C, 40.30; H, 2.99; N, 20.9%. Found: C, 42.73; H, 3.71; N, 24.9. Calcd. for $C_8H_8N_4O_4$ (acetaldehyde 2, 4-dinitrophenyl hydrazone): C, 42.86, H, 3.60; N, 25.1%].

The amount of pyruvate trapped as the semicarbazone is in relative proportion to the decrease of lactic acid and that of acetaldehyde in proportion to the decrease of ethanol formation. These results suggest that pyruvate seems to be an obligatory precursor of lactic acid, whereas ethyl alcohol formed in glucose fermentation may result from the reduction of acetaldehyde. Whether acetaldehyde detected as an intermediate is bound with a sulfhydryl group such as CoA in its active reduction to ethanol will be discussed later.

When ethanol is aerobically contacted with the resting cells of *L. fermenti* in phosphate buffer only a part of ethanol is consumed for several hours to give acetic acid, whereas if excess of semicarbazide is present, appreciable production of acetaldehyde is demonstrated as a direct intermediate to acetic acid (Table II). The similar results were observed in the manometric experiments (Fig. 1). These data show that oxygen uptake due to the utilization of the substrate is accelerated by the addition of large excess of semicarbazide, followed by the trapping of the simple precursor. After incubation, the contents of every Warburg cup was analyzed to estimate cumultive amounts of acetaldehyde as an intermediate. These data are presented in Fig. 1.

TABLE II. ACCUMULATION OF ACETALDEHYDE IN THE OXIDATION OF ETHANOL

Expt. No.	Ethanol utilized	Acetate formed	Acetaldehyde formed
	μmol	μmol	μmol
1	176	48	93
2	43	25	0
3	—	0	0

Reaction mixture contained: ethanol, 1 mmole; $MgCl_2$, 50 μmoles; tris buffer (pH 7.8), 200 μmoles; phosphate buffer (pH 8.0), 1.5 mmole; resting cells, 410 mg; semicarbazide, 1 mmole; in a total volume of 50 ml. Incubation was carried out aerobically at 30°C for 5 hours on a shaking incubator. Expt. 1, complete system; Expt. 2, without semicrrbazide; Expt. 3, without substrate.

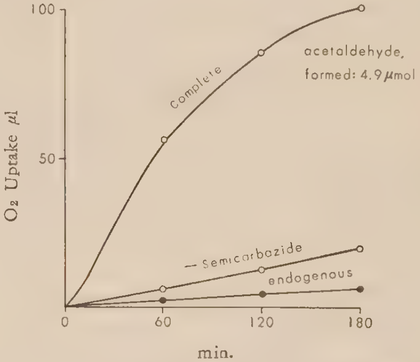


FIG. 1. Oxidation of Ethanol by Resting Cells of *L. fermenti*.

Each Warburg cup contained: 100 μmoles of phosphate buffer (pH 7.0), 5 μmoles of $MgCl_2$, 50 μmoles of ethanol, resting cells, 15 mg in a total volume of 2.3 ml. Gas phase, air; temperature, 37°C.

Although resting cells of *L. fermenti* can hardly utilize acetaldehyde anaerobically as a sole carbon source, it is observed that when acetaldehyde is added to the system, in which vigorous fermentation of glucose is proceeding, approximately one-third of added acetaldedyde

TABLE III. DISMUTATION OF ACETALDEHYDE TO ETHANOL AND ACETATE

Expt. No.	Substrate utilized (μmol)		End products (μmol)		
	Glucose	Acetaldehyde	Lactate	Ethanol	Acetate
1	970	374	915	1,045	310
2	1,000	—	930	883	134
3	—	18	37	0	0
4	—	—	42	0	0

Reaction mixture contained: phosphate buffer (pH 7.5), 250 μmoles; tris buffer (pH 7.8), 200 μmoles; glucose, 1 mmole; acetaldehyde, 1 mmole; $MgCl_2$, 50 μ moles; $MnSO_4$, 15 μmoles; $CaCO_3$, 200 mg; resting cells, 300 mg; in 85 ml. Static incubationw as carried out at 30°C for 5 hours. Expt. 1, complete system; Expt. 2, without acetaldehyde; Expt. 3, without glucose; Expt. 4, without both substrates.

TABLE IV. FORMATION OF ACETYL COMPOUNDS FROM ACETALDEHYDE

Reactant omitted	Acetohydroxamate formed (μmol)
None	2.4
TPN (or DPN)	0.7
CoA	0.8
Glutathione	1.5
Phosphate	1.2
Acetaldehyde	0.1
Extracts	0.4

Reaction mixture contained: tris buffer (pH 7.8), 40 μmoles; phosphate buffer (pH 8.0), 35 μmoles; dialyzed extracts, 0.5 ml; TPN (or DPN), 0.23 μmoles; CoA, 28 units; acetaldehyde 220 μmoles; glutathione, 24 μmoles; hydroxylamine, 100 μmoles; in 10 ml. Static incubation was carried out at 30°C for 3 hours.

disappears and that a dismutation of acetaldehyde to ethanol and acetate takes place (Table III).

Crude extracts of *L. fermenti* is able to utilize small amount of acetaldehyde, in the presence of TPN (or DPN), CoA, glutathione, orthophosphate and large excess of acetaldehyde (Table IV) and produce acetyl compounds (i.e. acetyl CoA or acetyl phosphate) as intermediates to acetic acid, which were detected and assayed after being derived to acetohydroxamate, which gave R_F 0.52 with solvent system; water-saturated butanol. These results indicate that several cofactors listed in Table IV would be necessary for the oxidation of acetaldehyde to acetate, since any anionic cofactors are not available in these extracts used for these experiments after treatment with Dowex-1 resin. Among these cofactors, TPN (or DPN) and CoA seem indispensable for the oxidation of the substrate, while glutathione appears to serve only for the reduction of CoA to CoASH, and inorganic phosphate for the conversion of acetyl CoA to acetyl phosphate.

The crude extracts of *L. fermenti* are also able to oxidize ethanol with the addition of large excess of semicarbazide. The enzyme preparation (ammonium sulfate fraction) also oxidize ethanol with the addition of either TPN or DPN. The results of the spectrophotometric experiment were revealed in Fig. 2 (A). Reversibly, reduced pyridine nucleotides were readily oxidized with the addition of acetyl CoA (Fig. 2 (B)). From these figures it would be visualized that the assayed fraction contained alcohol dehydrogenase, which seems to be non-specific for pyridine nucleotides, and aldehyde dehydrogenase which requires CoA. The reaction catalyzed with both enzymes are readily reversible in vitro; in Fig. 2 (A), after reading maximum absorption at 340 mμ, subsequent addition of acetyl phosphate and CoA to this system gives considerable decrease of the absorption due to the oxidation of reduced coenzymes, followed by the increase of the reduced product (acetaldehyde). In the contents of the Beckman cuvette after the reversible reaction, corresponding amount of acetaldehyde was detected in the complete system, compared with reference cuvette. It would be also demonstrable that acetyl CoA is stepwise reduced to ethanol via acetaldehyde in the presence of reduced coenzymes of *L. fermenti*. There results have good resemblance to those suggested in *E. coli* and in *Cl. kluyveri*. However, it would be also postulated that two different enzymes, TPN- and DPN-specific alcohol dehydrogenases seem to occur in the fraction of the enzyme.

Impossibility of ethanol formation from pentoses by heterofermentative bacteria means requisite of an oxidative system to be coupled with the reduction of acetyl phosphate which is derived from ketopentose phosphate. There-

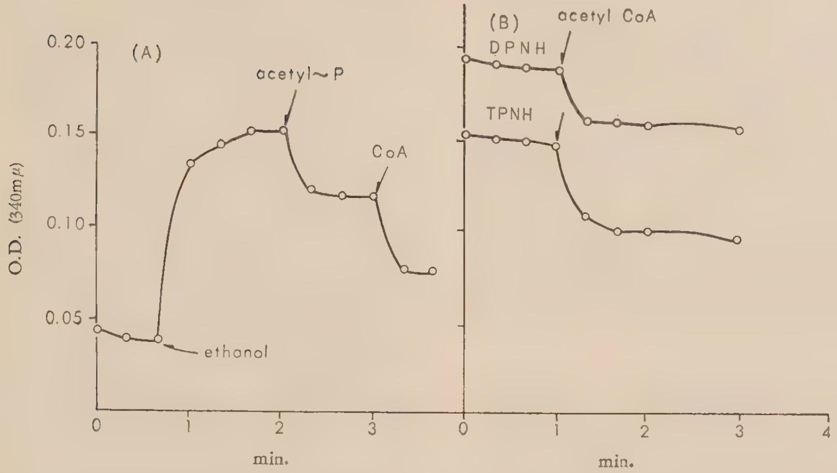


FIG. 2 (A). Coupling of Alcohol Dehydrogenase with Aldehyde Dehydrogenase.

Reaction mixture contained: tris buffer (pH 8.2), 150 μmoles; phosphate buffer (pH 8.0), 20 μmoles; TPN (or DPN), 0.25 μmoles; semicarbazide, 20 μmoles; enzyme fraction, 0.05 ml, in a total volume of 3.5 ml. Subsequent additions were 200 μmoles of ethanol, 25 μmoles of Li_2-acetyl phosphate and 2.2 units of CoA plus 28 μmoles of glutathione, in order. Change of optical density was read before and after addition of substrate at 20 second-intervals in a Beckman cuvette with the light path of 1.0 cm.

(B) Reduction of acetyl CoA in the presence of pyridine nucleotides. Reaction conditions were the same as described in (A), except that subsequent addition was 17 μmoles of acetyl CoA.

fore, it would be reasonable to presume that if a synchronizable oxidation system or an appropriate electron donor co-exists with pentose dissimilation by heterofermentative microorganisms ethanol formation from pentoses could, in an optimal environment, be realized.

1. Coupling with mannitol oxidation.

As indicated in the previous paper, heterolactic fermenters are readily able to oxidize mannitol to fructose in an aerobic condition. With this standpoint, an assumption could be possible that a couple of electron to be transport, in aerobic experiments, up to air oxygen may be available anaerobically for the reduction of acetyl CoA when D-xylose is simultaneously utilized with the same organism. In other words, this attempt aims to actualize a reverse reaction of D-mannitol production by heterofermentative lactobacilli. *Leuc. mesenteroides* and *L. fermenti* which belong to the strain of mannitol-producible, xylose-fermentable heterofermenter. The results shown in Table V illustrate that mannitol utilization by these organisms was rather difficult in semi-anaerobic (static) incubation, but with the addition of xylose, appreciable amount of mannitol was consumed, and that detectable increase of ethanol formation is manifested in the latter case (Expts. 3 and 6). It would be conceivable that increased amount of ethanol beyond its expected molarity should be due to the transformation of acetyl phosphate from xylose to ethanol.

TABLE V. SIMULTANEOUS UTILIZATION OF MANNITOL AND D-XYLOSE BY HETEROFERMENTATIVE MICROORGANISMS

Expt. No.	xylose utilized	mannitol utilized	products		
			lactate	ethanol	acetate
	mmol	mmol	mmol	mmol	mmol
1	1.00	—	0.92	—	1.05
2	—	0.08	0.06	0.07	0.00
3	1.00	0.37	1.22	0.60	0.67
4	1.00	—	0.88	—	1.12
5	—	0.12	0.09	0.09	0.00
6	1.00	0.28	1.16	0.48	0.83

Each flask contained: 1 mmole of mannitol, 1 mmole of xylose, 2 mmoles of phosphate buffer (pH 7.5), 45 μmoles of $MgCl_2$, 17 μmol of $MnCl_2$, 1.7g of resting cells of *L. fermenti* (Expts. 1,2 and 3) and of *Lenc. mesenteroides* (Expts. 4,5 and 6), in 50 ml. Incubation was carried out statically for 6 hours at 37°C. Expt. 1 and 4, without mannitol; Expt. 2 and 5, without xylose; Expt. 3, 6, complete system.

More distinct evidence was obtained with the

TABLE VI. SIMULTANEOUS UTILIZATION OF MANNITOL AND L-ARABINOSE BY HOMOFERMENTATIVE MICROORANISM

Expt. No.	L-arabinose utilized	mannitol utilized	products		
			lactate	ethanol	acetate
	mmol	mmol	mmol	mmol	mmol
1	0.50	—	0.53	0.02	0.60
2	—	0.16	0.30	0.03	0.15
3	0.50	0.17	0.75	0.31	0.35

Experimental conditions were the same as described in Table V, except that 0.5 mmole of L-arabinose and 1.5g of resting cells of *L. arabinosus* were used. Expt. 1, without mannitol; Expt. 2, without arabinose; Expt. 3, complete system.

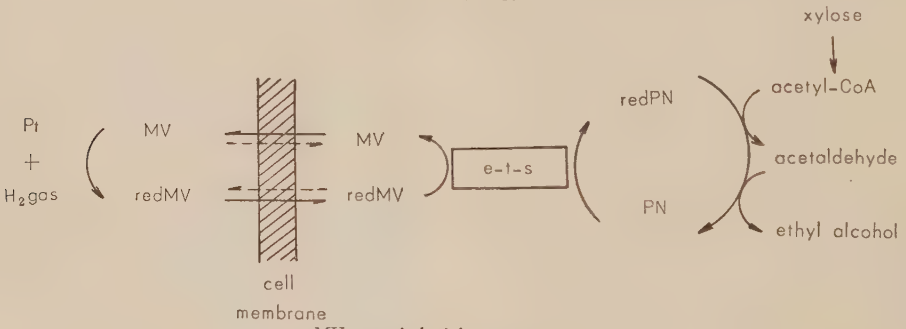
experiments using *L. arabinosus* which belongs to the strain of D-mannitol-utilizable, L-arabinose-fermentable homofermenter (Table VI). Only trace amount of ethanol is produced from each separated substrate, but when simultaneous occurrence of both substrate (Expt. 3) happened in the incubated broth, large amount of ethanol is produced probably because a couple of electron liberated with the dehydrogenation of mannitol-1-phosphate to fructose-6-phosphate serves in vivo for the reduction of acetyl phosphate derived from pentose phosphate. Satisfactory decision to justify these presumption will be achieved only by a series of works using xylose-1-C^{14}.

2. Supply of reduced methyl viologen.

At the first stage, methyl viologen (E_0': -446 mV) undergoes extracellular reduction with platinum asbestos in hydrogen gas phase, then the reduced dye penetrates the cell membrane of microorganism, as a donor of electron, which is finally accepted with ethanol precursors mediated pyridine nucleotides (E_0': -320 mV). For good results two indispensaple factors must be controlled; one is that successive and stable reduction of oxidized methyl viologen takes place without serious inhibition on other internal redox systems, and the other is that adequate permeability for the reduced dye is gifted to the bacterial cell membrane.

From view point of practical technique, an absolutely anaerobic gas phase was required for the infinite production of reduced methyl viologen in circulation. Another important factor is the adjustment of pH which shows direct effect on the stability of the reduced dye. In a 500 ml-round bottom flask with a rubber stopper, through which an outlet and an inlet glass tube for the gas exchange were installed, the reaction mixture containing cell suspension, substrate, dye and platinum asbestos was incubated in a shaking incubator at 30°C or continuously stirred on a magnetic stirrer, in hydrogen gas phase. The pH of the contents was adjusted with dropwise addition of 0.1 N NaOH in order to to decolorize blue tint of the reduced dye. After an expected period of incubation elapsed, the contents were analyzed.

DIAGRAM 1.



MV: methyl viologen
redMV: reduced methyl viologen
PN: pyridine nucleotides
redPN: reduced pyridine nucleotides
e-t-s: electron transfer system

TABLE VII. ETHANOL FORMATION FROM D-XYLOSE BY RESTING CELLS OF *L. fermenti* AND *L. Xylosus*

Expt. No.	xylose utilized	products		
		lactate	acetate	ethanol
	mmol	mmol	mmol	mmol
1	0.86	0.78	0.89	trace
2	0.72	0.63	0.42	0.26
3	0.42	0.33	0.23	0.31
4	0.76	0.68	0.83	trace
5	0.51	0.44	0.32	0.14
6	0.53	0.42	0.35	0.11

Reaction mixture contained: 2 mmoles of phosphate buffer (pH 7.5), 3 mmoles of tris buffer (pH 7.8), 1 mmole of xylose, 20 μmoles of methyl viologen chloride, 10 mg of platinum asbestos, 25 μmoles of $MgCl_2$ and 1.2 g of resting cells of *L. fermenti* (Expts. 1,2 and 3) or 1.5 g of resting cells of *L. xylosus* (Expts. 4,5 and 6); in 50 ml. The mixture was stirred by a magnetic stirrer under hydrogen gas phase for 5 hours. In Expts. 1 and 4, methyl viologen was not added.

TABLE VIII. ETHANOL FORMATION FROM L-ARABINOSE BY RESTING CELLS OF *L. mesenteroides* AND *L. arabinosus*

Expt. No.	arabinose utilized	products		
		lactate	acetate	ethanol
	mmol	mmol	mmol	mmol
1	0.83	0.76	0.92	0
2	0.56	0.44	0.61	0.07
3	0.39	0.32	0.45	0.05
4	0.55	0.49	0.51	0
5	0.48	0.44	0.45	trace
6	0.33	0.29	0.38	trace

Experimental conditions were the same as described in Table VII, except that 1.15 g of resting cells of *Leuc. mesenteroides* (Expts. 1,2 and 3) or 2.0 g of resting cells of *L. arabinosus* (Expts. 4,5 and 6) was added. Expts. 1 and 4, without methyl viologen.

Some typical results among many data are presented in Table VII.

Although complete disappearance of volatile acid was not yet practicable, the data in Table VII indicate that approximately half amount of acetic acid, if normally, to be produced seems to have been transformed into ethyl alcohol. Thus this mode of fermentation appears actually corresponding, except evolution of carbon dioxide, with that of gluconate fermentation by the same organism in anaerobic conditions:

$$\text{Xylose} + \text{red MV}^{*} \rightarrow \text{Lactate} + \tfrac{1}{2}\ \text{Acetate} + \tfrac{1}{2}\ \text{Ethanol} + \text{ox MV}^{**}$$

$$\text{Gluconate} \rightarrow \text{Lactate} + \tfrac{1}{2}\ \text{Acetate} + \tfrac{1}{2}\ \text{Ethanol} + CO_2 + H_2O$$

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* reduced methyl viologen
** oxidized methyl viologen

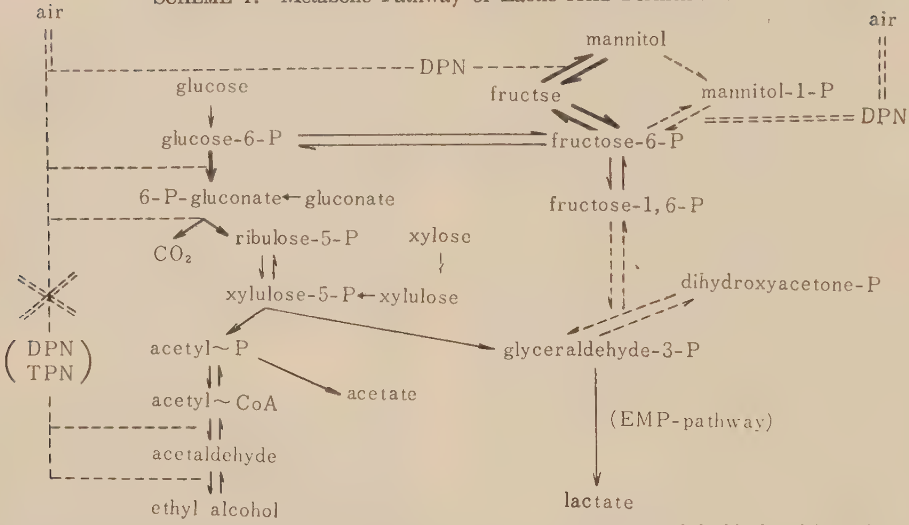
On the contrary, the results in Table VIII show that ethyl alcohol is poorly produced from arabinose even by the microorganisms which can readily utilize arabinose in anaerobic circumstances. Satisfactory interpretation to the difficulty of ethanol formation from arabinose is still impossible though it would be conceivable that also with respect to redox system of *L. arabinosus* participating in ethanol formation (alcohol dehydrogenase, aldehyde dehydrogenase and phosphotransacetylase) might be enough active to produce ethanol from acetyl phosphate (Table VI).

DISCUSSION

In the normal heterolactic fermentation it seems to be difficult to detect acetaldehyde as an intermediate of ethanol formation because of its rapid reduction to ethanol without transient extracellular accumulation of acetaldehyde. With the addition of semicarbazide, however, pyruvate and acetaldehyde, even during anaerobic fermentation of glucose, are accumulated instead of their reduction of lactate and ethanol, respectively, the quantities of which are sufficient to be recovered for estimation. This valuable view would extend the knowledge of quantitative investigation of the products formed by heterofermentative microorganisms.

With respect to coenzyme dependence, it has been reported that, in ethanol formation from acetyl CoA in the extract of *Cl. kluyveri* or *E. coli*, reduced DPN is absolutely required. On the contrary, in *L. fermenti* alcohol and aldehyde dehydrogenases appear to have no strict specificity for pyridine nucleotides, although CoA-dependent reduction of acetyl phosphate to acetaldehyde proves to be more accelerated with TPN than with DPN. The presumption of the occurrence of two different dehydrogenases (DPN- and TPN-linked) might well be ruled out since glucose-6-phosphate and 6-phosphogluconate dehydrogenases, which should couple with alcohol- and aldehyde dehydrogenases in vivo, are also found to be non-specific for pyridine nucleotides of the same microorganism. Further clarification of these phenomena still

SCHEME 1. Metabolic Pathway of Lactic Acid Fermentation



Single dotted lines represent coupling system of dehydrogenases (anaerobic fermentation), and double dotted lines electron transport to air oxygen (aerobic fermentation). Bold arrow heads indicate metabolic routes characteristic of heterofermentative microorganisms, dotted arrow heads routes characteristic of homofermentative microorganisms, and fine arrow heads common routes to both species.

remains to be researched. Summarizing many data and discussions, the authors suggest a reasonable metabolic pathway of lactic acid fermentation in which elucidation for the accumulation and utilization of mannitol by lactic acid bacteria is included (Scheme I).

With the procedures for the experiments using methyl viologen more satisfactory results would not be obtained unless velocity of pentose degradation efficiently slides in continuous reproduction-cycle of reduced methyl viologen, linking between extra- and intracellular metabolic pools. Besides this difficulty, another problem would await resolution that other electron transfer systems contained in the microorganisms are checked by descendence of redox potential towards a degree of platinum electrode which is continuously functioning in a fermentation broth. Thereby these two main barriers, above pointed out, would obstruct prospective application of the dye method to industrial region.

SUMMARY

1. Pyruvate and acetaldehyde were detected as intermediates in the pathway of heterolactic fermentation by *L. fermenti*.

2. Acetaldehyde was accumulated aerobically as an oxidation product of ethanol in the presence of semicarbazide.

3. Dismutation of acetaldehyde to ethanol and acetate was observed when acetaldehyde was added to the system of glucose fermentation.

4. Alcohol dehydrogenase in an ammonium sulfate fraction catalyzes both DPN- and TPN-dependent oxidation of ethanol to acetaldehyde. Acetyl CoA was reduced in the presence of either TPN or DPN with the same fraction.

5. By coupling of D-xylose degradation with mannitol utilization appreciable increase of ethyl alcohol was observed with resting cells of heterolactic microorganisms.

6. With the continuous supply of reduced methyl viologen approximately half molarity of acetate to be produced from D-xylose was transformed to ethyl alcohol in an absolutely anaerobic condition. From L-arabinose only poor amount of ethyl alcohol was produced by both strains of microorganisms.

7. A metabolic pathway of lactic acid fermentation including D-mannitol fermentation was suggested.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 595~597, 1960]

Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part VIII. On the Reduction of (−)-Isomenthone*

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Received March 17, 1960

In the reduction of (−)-isomenthone with sodium in aqueous ammonia, it was found that the reduction product gives a mixture consisting of 75.5% of (−)-isomenthol (a,e,e), 9.5% of (+)-menthol (e,e,e) and 8.6% of (−)-neoisomenthol (a,a,e). From this fact, it might be concluded that this reduction is stereospecific for isomenthone and is mechanistically different from reduction with sodium and alcohol.

In the previous papers of this series, it has been shown that (−)-menthone is reduced quantitatively to (−)-menthol by treatment with sodium in aqueous ammonia[1], and a small amount of the by-product (ca. 10%) has been presumed to be (+)-neomenthol. However, from the result of elution gas-chromatographic study by Tagaki[2], it was found that this by-product is not (+)-neomenthol but (+)-isomenthol, and therefore, the conclusion previously reported should be revised showing that (−)-menthone is reduced with sodium in aqueous ammonia to (−)-menthol (90%) and (+)-isomenthol (10%)

On the other hand, by application of this method of reduction, (+)-isomenthol has been obtained in a good yield from inverted menthone, namely a mixture of (−)-menthone and (+)-isomenthone[3]. In this case, the (+)-isomenthol content in menthol mixture obtained from the reduction of inverted menthone was lower than the theoretical value which was expected from the (+)-isomenthone content in the original inverted menthone. As an explanation for this fact, it seemed reasonable to assume that (+)-isomenthone is considerably inverted to (−)-menthone by alkali, while (−)-menthone is hardly inverted to (+)-isomenthone.

The present investigation was undertaken in order to clarify this mechanistic problem.

(−)-Isomenthone was reduced with sodium in aqueous ammonia, and the compositions of isomerides in the resulted product were investigated. The application of a combination of recrystallisation and absorption chromatography revealed that the reduction product of (−)-isomenthone consisted of 75.5% of (−)-isomenthol (a,e,e), 9.5% of (+)-menthol (e,e,e) and 8.6% of (−)-neoisomenthol (a,a,e). In this case no (−)-neomenthol was identified. The production of neoisomenthol may be related somewhat to the fact that isomenthone is reduced predominantly to neoisomenthol with lithium aluminum hydride[2].

The difference of isomenthol content from the theoretical value described above might be attributed to the production of neoisomenthol from isomenthone and also to the inversion of isomenthone to menthone.

It is interesting to note that the reduction of isomenthone with sodium in aqueous ammonia afforded isomenthol in predominance, while with sodium and alcohol, menthol predominated[4].

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* For Part VII see This Bulletin, **24**, 508 (1960).

[1] H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).

[2] Y. Tagaki, private communication.

[3] H. Ueda and S. Shimizu, This Bulletin, **23**, 380 (1959).

EXPERIMENTAL

All melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto. Infrared spectra were recorded with a Shimadzu spectrophotometer.

(−)-Isomenthone (−)-Isomenthone fraction (19.1 g), b.p. 66~68°/6 mm, d_4^{23} 0.8988, n_D^{23} 1.4532, $[\alpha]_D^{23}$ −73.9° (homog.), obtained from geranium oil (*Pelargonium graveleus*) by fractional distillation, was reduced with sodium in aqueous ammonia according to the procedure previously reported[1]. The product (17.0 g), b.p. 73~75°/4.5 mm, was recrystallised from nitromethane to give pure (−)-isomenthol (8.1 g), m.p. 81.0~81.8°. The resulting (−)-isomenthol (7.00 g) was treated with Beckmann's chromic acid at 47° to give pure (−)-isomenthone (6.12 g, Yield 88.56%), b.p. 67.0°/6 mm, d_4^{17} 0.9039, n_D^{17} 1.4544, MR 46.24 (Calcd. 46.19), $[\alpha]_D^{17}$ −94.0° (homog.).

Reduction of (−)-Isomenthone (−)-Isomenthone (5.33 g) was reduced with sodium (4.0 g) in aqueous ammonia and benzene, according to the procedure previously described[1]. The reaction temperature was maintained in the range of 0~3°. The product was treated with semicarbazide hydrochloride in the same way as previously reported[3]. The unreacted menthone was completely converted into semicarbazone (0.36 g) and removed by steam distillation. The fraction, from which the contaminated menthone was removed, was distilled to give a menthol mixture (4.77 g, Yield 88.44 % based on (−)-isomenthone), b.p. 75~77°/5 g mm, m.p. 55~65°, $[\alpha]_D^{17}$ −8.77° (c, 19.378 in ethanol).

Separation of Menthol Isomers Recrystallisation of the menthol mixtvre (4.72 g) from *n*-hexane gave (−)-isomenthol (3.21 g, Yield 68.07% from menthol mixture), m.p. 81.2~81.9°. It gave the corresponding 3,5-dinitrobenzoate as pale yellow needles, m.p. 146.5~147.0°; *Anal.* Found: C, 58.55; H, 6.52. Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%.

Esterification of the material recovered from the mother liquors of the above-crystallisations of the menthol mixture gave crude 3,5-dinitrobenzoate (2.91 g, Yield 86.04% for theor.). Careful fractional recrystallisations of this ester from *n*-hexane gave the following fractions: (1) Ester-I, m.p. 127°, 1.20 g (Recovery 41.47% from crude ester); (2) Ester-II, m.p. 105~112°, 0.12 g (3.98%); (3) Ester-III, m.p. 87~89°, 1.12 g (38.60%); (4) Ester-IV, m.p. 54~62°, 0.21 g (7.32%); the experimental loss being 0.25 g (8.61%).

TABLE I. ABSORPTION CHROMATOGRAPHY OF ESTER-I (m.p. 127°)

Fraction No.	Eluted Solvent Total	Recovered Material Weight	Recovered Material Melting point	
	ml	mg	°C	
1~9	90	0	—	
10	100	0.6	153.5~154.0	(+)-Menthyl 3,5-dinitrobenzoate
11	110	1.6	153.5~154.0	
12	120	2.1	153.5~154.0	
13	130	2.7	153.5~154.0	
14	140	2.9	150.0~150.5	
15	150	1.7	127~128	Mixture
16	160	2.1	126~127	
17	170	1.9	132~133	
18	180	1.0	137~138	
19*	190	8.2	145.0~145.5	(−)-Isomenthyl 3,5-dinitrobenzoate
20*	200	2.8	145.0~145.5	
21~27*	270	0	—	
	Total	27.6 mg (Recovery 92%)		

* Fractions 19~27 were eluted by 5% ether-*n*-hexane.

(1) Ester-I, m.p. 127°, $[\alpha]_D^{20}+19.68°$ (c, 11.028 in chloroform), was not altered on further recrystallisations. *Anal.* Found: C, 58.56; H, 6.37. Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%. From optical rotation, it probably consists of (±)-menthyl 3,5-dinitrobenzoate. In order to make a more detailed study of the composition, Ester-I (30.0 mg) was eluted by *n*-hexane on an alumina column (Brockmann Alumina, 9.0 g; Column diameter, 11 mm), and it gave two fractions, as shown in Table I. However, no other fraction was obtained. These fractions were identified by the mixed m.p. comparison with authentic samples.

From the result of chromatography, Ester-I seemed to be composed of approx. 45.7% of (+)-menthyl 3,5-dinitrobenzoate and 54.3% of (−)-isomenthyl 3,5-dinitrobenzoate. This proportion agreed well with values of 43.6% and 56.4%, calculated from the optical rotation of Ester-I.

(+)-Menthyl 3, 5-dinitrobenzoate, m.p. 153°, used in the mixed m.p. method, was prepared from (−)-isomenthone by reduction with sodium and alcohol.

(2) Ester-II, m.p. 105~112°, probably consists of a mixture of Ester-I and Ester-III, but its composition

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[4] J. Read and A.M.R. Cook, *J. Chem. Soc.*, **1925**, 2782.

TABLE II. ABSORPTION CHROMATOGRAPHY OF ESTER-III (m.p. 87~89°)

Fraction No.	Eluted Solvent Total ml	Recovered Material		
		Weight mg	Melting point °C	
1~11	110	0	—	
12	120	1.1	153.0~153.5	(+)-Menthyl 3,5-dinitrobenzoate
13	130	2.4	153.0~153.5	
14	140	2.7	130~131	Mixture
15	150	3.3	88~90	
16	160	2.4	87~89	
17	170	2.1	91~93	crude. (−)-Neoisomenthyl 3,5-dinitrobenzoate
18	180	2.1	91~94.5	
19	190	1.9	91~94.5	
20	200	1.9	91~93	
21	210	1.5	91~93	
22	220	1.3	91~92	
23	230	1.0	91~92	
24	240	0.9	90	
25~29	290	0	—	

Total 25.9 mg (Recovery 86%)

was not determined.

(3) Ester-III, m.p. 87~89°, $[\alpha]_D^{20}+17.25°$ (c, 10.726 in chloroform), could not be purified satisfactorily by recrystallisation. It probably consists of a mixture of 3,5-dinitrobenzoates of menthol isomers by elementary analysis. *Anal.* Found: C, 58,78; H, 6.42, Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%. Then, Ester-III (30.0 mg) was chromatographed on alumina, in the same way as described above, to give two fractions, m.p. 153.5° and m.p. 91~94.5°, as shown in Table II. The former fraction was identified to be (+)-menthyl 3,5-dinitrobenzoate by the mixed m.p. method. Crystallisation of the latter fraction (8.0 mg) from methanol gave pale yellow needles (4.7 mg), m.p. 92~93.5°, mixed m.p. 96~98° with (−)-neoisomenthyl 3,5-dinitrobenzoate, m.p. 99.5~100°. The infrared spectrum of this substance (in nujol) agreed completely with that of (−)-neoisomenthyl 3,5-dinitrobenzoate.

This experimental result shows that Ester-III consists of approx. 30.1% of (+)-menthyl 3,5.dinitrobenzoate and 69.9% of (−)-neoisomenthyl 3,5-dinitrobenzoate. This proportion also agreed well with values of 30.1% and 69.9%, calculated from the optical rotation of Ester-III.

The authentic sample of (−)-neoisomenthyl 3,5-dinitrobenzoate, m.p. 99.5~100°, was prepared from (−)-isomenthone by reduction with lithium aluminum hydride.

(4) Ester-IV, m.p. 54~62°, was not further investigated because of the small quantity available.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 598~600, 1960]

Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part IX. On the Reduction of (±)-Piperitone*

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In the reduction of (±)-piperitone with sodium in aqueous ammonia, by means of infrared analyses, it was found that the reduction product is composed of (±)-isomenthol in a slight excess over (±)-menthol, and the components were isolated in a pure state.

The reduction of (±)-piperitone has been studied by several groups of workers. Hughdesdon, Smith and Read¹⁾ reported that (±)-piperitone is reduced with sodium and alcohol to give a menthol of m.p. 39~41°. The fact that the m.p. of (±)-menthol is 38°, and that of (±)-isomenthol is 53.5° seems to indicate that this menthol is mainly composed of (±)-menthol. Read and Cook²⁾, however, isolated (±)-isomenthone as semicarbazone in a slight excess over (±)-menthone from the oxidation product of this menthol. Misrock and Church³⁾ reported that (±)-piperitone dissolved in diisopropyl carbinol and benzene is reduced with sodium dispersed in iso-octane to give menthol in a quantitative yield. The menthol was oxidised to menthone which was identified from its refractive index to be composed of approx. 77% of (±)-isomenthone and 23% of (±)-menthone.

So far, no isolation of menthol isomers from the reduction product of (±)-piperitone has been accomplished.

In the previous papers, it has been shown that (−)-menthol is obtained predominantly from (−)-menthone⁴⁾, (+)-pulegone⁵⁾ and (+)-menthone enol acetate⁶⁾ by treatment with sodium in aqueous ammonia. Also, (−)-dihydrocarveol is prepared from (−)-carvone⁷⁾ in the same manner of reduction. Both reduction products are of the same conformation (e,e,e).

The present study was undertaken in order to determine the composition of the reduction product of (±)-piperitone, and to isolate the resulting menthol isomers.

The reduction product of (±)-piperitone with sodium in aqueous ammonia was shown to consist of alcohols (90%) and carbonyl compounds (10%). (±)-Isomenthone semicarbazone was isolated from the carbonyl fraction. (±)-Menthol and (±)-isomenthol were isolated from the alcohol fraction by recrystallisation and chromatography of 3,5-dinitrobenzoate. Although the production of (±)-neoisomenthol was expected from the result of reduction of (−)-isomenthone⁸⁾, this could not be obtained. Infrared analyses and chromatographical data, when combined with those of the reduction of (−)-isomenthone⁸⁾ and (±)-isomenthone⁹⁾, show-

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* For Part VII see This Bulletin, **24**, 595 (1960).

1) R.S. Hughesdon, H.G. Smith and J. Read, *J. Chem. Soc.*, **1923**, 2916.

2) J. Read and A.M.R. Cook, *J. Chem. Soc.*, **1925**, 2782.

3) S.L. Misrock and J.M. Church, *Ind. Eng. Chem.*, **49**, 823 (1957).

4) H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).

5) H. Ueda and S. Shimizu, This Bulletin, **24**, 92 (1960).

6) H. Ueda, This Bulletin, **24**, 508 (1960).

7) H. Ueda and S. Shimizu, This Bulletin, **23**, 524 (1959).

8) H. Ueda, This Bulletin, **24**, 595 (1960).

9) H. Ueda, This Bulletin, **24**, 506 (1960),

ed that (±)-piperitone yields (±)-isomenthol in a slight excess over (±)-menthol by reduction with sodium in aqueous ammonia.

It seems to be noteworthy to see how isomenthol (a,e,e) but not menthol (e,e,e), unlike pulegone, could be the major product formed in the reduction of piperitone.

EXPERIMENTAL

All melting and boiling points were uncorrected. Infrared spectra were recorded at Prof. Nakajima's Laboratory, University of Kyoto. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto. Ultraviolet absorption spectra were determined at Prof. Shimizu's Laboratory, Shinshu University.

(±)-Piperitone From high boiling point fraction of Japanese peppermint oil, (±)-piperitone was obtained by treatment with sodium sulfite; b.p. 98~100°/9.5 mm, d_4^{20} 0.9418, n_D^{20} 1.4864, MR 46.44 (Calcd. 45.72), $[\alpha]_D^{20}-1.6°$ (homog.), λ_{max} 235 mμ, E_{max} 14621 (in methanol), 2,4-dinitrophenylhydrazone m.p. 113.5~114.5°.

Reduction of (±)-Piperitone (±)-Piperitone (10.0 g) was reduced with sodium (8.3 g) in aqueous ammonia and benzene, according to the procedure previously reported[4]. The reaction temperature was maintained at 0~3°. After treatment in the usual manner, the reduction product (7.9 g, Yield 77.0%) was obtained by distillation, as a colorless semi-solid possessing a pleasant odor; b.p. 75.7~76.5°/4 mm, λ_{max} 235 mμ, E_{max} 790 (piperitone content 5.4%). The carbonyl content was 10.4% according to the hydroxylamine method[4].

The distillation residue (2.0 g) was gradually crystallised after standing for a few days at room temperature. It was probably pinacone*, which was not further investigated because of the small amount available.

Separation of Carbonyl Compound (By-product) The resulting reduction product (6.7 g) was treated with semicarbazide hydrochloride, according to the procedure reported[10]. The carbonyl compound was completely converted into semicarbazone, and separated from the unchanged fraction by steam distillation. Recrystallisation of crude semicarbazone (558 mg), m.p. 150~186°, gave (±)-isomenthone semicarbazone (77 mg), m.p. 218°, λ_{max} 228 mμ, E_{max} 14300 (in methanol). Its m.p. was undepressed by admixture with an authentic sample. An easily soluble semicarbazone (189 mg), m.p. 150~166°, λ_{max} 229 mμ, E_{max} 13500 (in methanol), obtained from the mother liquor, seemed to be a mixture of (±)-menthone semicarbazones.

Menthol Mixture Distillation of the fraction, from which the contaminated menthone was removed, gave a menthol mixture (5.9 g), b.p. 72~72.5°/3 mm, m.p. 28~30°, which was identified as to be mainly a mixture of (±)-menthol and (±)-isomenthol by its infrared spectrum. Any absorption band attributed to the presence of other isomerides was not shown in this spectrum. It was found that the menthol mixture consisted of approx. 45% of (±)-methol and 55% of (±)-isomenthol by comparison of its infrared spectrum with those of mixtures of various compositions of (±)-menthol and (±)-isomenthol (key bands, 967 and 1101 cm^{-1}).

Separation of Menthol Isomers

(1) The menthol mixture (9.34 g) was recrystallised three times from *n*-hexane. The product (3.32 g) had m.p. 34.3~37.9° (see below). The recovered material (5.71 g) from mother liquors in case of the above recrystallisations was esterified to give crude *p*-nitrobenzoate (7.62 g) as yellow viscous oil and unesterified oil (0.67 g). Six crystallisations of the crude ester from methanol gave pure (±)-menthyl *p*-nitrobenzoate (1.57 g), m.p. 91~91.5°, undepressed by admixture with an authentic sample.

The product, m.p. 34.3~37.9°, described above, was further recrystallised six times from nitromethane to give (±)-isomenthol (0.75 g), m.p. 50.0~51.2°, mixed m.p. 50.5~51.2° with an authentic sample, m.p. 52~53°. (±)-Isomenthol gave the corresponding 3,5-dinitrobenzoate, m.p. 129~129.7°, undepressed by admixture with an authentic sample.

(2) The menthol mixture (5.31 g) was esterified with 3,5-dinitrobenzoylchloride in pyridine. The product (11.03 g, Yield 92.6%) was crystallised twice from methanol to give 3,5-dinitrobenzoate (Ester-I) (8.44 g, Yield 76.6% from crude ester), m.p. 121°; *Anal.* Found: C, 58.62; H, 6.27. Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%. Mixed m.ps. with (±)-menthyl, (±)-isomenthyl and (±)-neomenthyl 3,5-dinitrobenzoate were 122~122.5°, 120~121° and 108~111°, respectively. This m.p., also, cor-

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[10] H. Ueda and S. Shimizu, This Bulletin, **23**, 380 (1959).

* On the reduction of (+)-camphor by the same method as described above, the product consisted of 74% of borneols, m.p. 201~203°, $[\alpha]_D^{25}+14.89°$ (in methanol), and 26% of camphor pinacone, m.p. 157~158°, $C_{20}H_{34}O_2$. It was shown that this reduction is somewhat accompanied by bimolecular reduction.

TABLE I. ABSORPTION CHROMATOGRAPHY OF ESTER-I (m.p. 121°)

Fraction No.	Eluted Solvent Total ml	Recovered Material Weight mg	Recovered Material Melting point °C	
1~9	90	0	—	
10	100	0.7	126.5~127.0	
11	110	1.7	127.0~128.0	(±)-Menthyl 3,5-dinitrobenzoate
12	120	2.5	128.0~128.5	
13	130	3.1	127.0~127.5	
14	140	2.1	126.5~127.5	
15	150	2.6	121	
16	160	1.8	119	
17	170	1.3	118.0~118.5	Mixture
18	180	1.6	115	
19	190	1.5	122.0~123.0	
20	200	1.5	126.0~127.0	
21	210	1.2	127.0~127.5	
22	220	1.0	128.0~128.5	
23	230	0.8	128.5~129.0	
24	240	1.0	129.5	(±)-Isomenthyl 3,5-dinitrobenzoate
25	250	0.4	129.5	
26	260	0.3	129.5	
27	270	0.2	129.5	
28*	280	2.9	128.0~128.5	
29*	290	0	—	

Total 28.2 mg (Recovery 96%)

* Fractions 28 and 29 were eluted by ether.

responded well with that reported for the substance, m.p. 121°, prepared by the reduction of (±)-isomenthone[9], and mixed m.p. was not depressed.

Regenerated alcohols, m.p. 37.7~38.0°, prepared from Ester-I, was identified to be a mixture of (±)-menthol and (±)-isomenthol by comparison of its infrared spectrum with those of known samples.

In order to make a more detailed study of the composition, Ester-I (30.0 mg) was eluted by *n*-hexane on alumina column (Brockmann Alumina, 9.0 g; Column diameter, 11 mm) and gave two fractions, (±)-menthyl and (±)-isomenthyl 3,5-dinitrobenzoates, as shown in Table I. No other fraction was obtained. These fractions were identified by mixed m.p. comparison.

This chromatographical data showed that Ester-I is composed of approx. 59% of (±)-menthyl 3,5-dinitrobenzoate and 41% of (±)-isomenthyl 3,5-dinitrobenzoate.

From the moter liquors in recrystallisations of the crude ester, Ester-II (2.26 g), m.p. 65~87°, was recovered, and this ester could not be purified satisfactorily by further recrystallisations. An attempt to determine its composition by means of chromatography was not successful.

Acknowledgements The author is indebted to Emeritus Prof. S. Takei, Prof. T. Mitusi, Prof. M. Nakajima, Dr. Inouye, University of Kyoto, and Prof. S. Shimizu, Shinshu University, for their kind guidance throughout this work. He also wishes to express his sincere thanks to Mr. K. Fujimoto and Miss. Y. Fukumoto for their assistance in carrying out this work.

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Studies on the Reduction of Terpenes with Sodium in Aqueous Ammonia

Part X. Synthesis of Menthol from Acetone and Formaldehyde*

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On the reduction of the condensation product of mesityloxide with methylvinylketone by treatment with sodium in aqueous ammonia, the main product was (±)-menthol and (±)-isomenthol, which were isolated in pure state. This experimental result suggests the possibility of menthol synthesis from acetone and formaldehyde.

Piperitenone has been isolated by Naves[1] from the essential oil of *Mentha Pulegium var. Villona Benthe.* He obtained acetone and levulinic acid on ozonolysis, acetone and 1-methyl-1-cyclohexene-3-one on digestion with formic acid, and thymol on mere heating with palladium. Naves and Papazian[2] also obtained menthols, mainly isomenthol, by hydrogenation over platinum oxide, while with a Raney nickel isomenthone was mainly obtained.

Piperitenone was also prepared from verbenone by pyrolysis[3].

Recently, Bergmann and Bracha[4] published the synthesis of piperitenone involving the condensation of mesityloxide with methylvinylketone. They obtained 1-methyl-1-cyclohexene-3-one and acetone on decomposition of its piperitenone by boiling formic acid, and thymol on transformation by heating with palladium. However, Bergmann's piperitone is not identical with those reported by Naves and other authors[5~9].

In the previous papers, it has been shown that carbonyl group and double bond conjugated with the carbonyl group in α,β-unsaturated ketone, such as carvone[10], pulegone[11] and piperitone[12], are reduced to saturated alcohol by treatment with sodium in aqueous ammonia, i.e., a novel method of reduction employed in this series.

The present investigation was undertaken to examine the composition of the reduction product of Bergmann's piperitenone.

In view of the above experimental results on the reduction of α,β-unsaturated ketone, if Bergmann's piperitenone is a cross conjugated ketone as well as the previous piperitenone, it is presumed that a carbonyl group and one of the two double bonds conjugated with the carbonyl group in piperitenone would be reduced with sodium in aqueous ammonia to give either pulegol or piperitol, which, in turn, would give menthols by catalytic hydrogenation, as reported by Macbeth[13].

In contrast to this expectation, however, the experiment showed that the reduction product was confirmed to be saturated alcohols, mainly (±)-menthol and (±)-isomenthol, by the application of the combination of infrared analyses and chemical methods. (±)-Menthyl and (±)-

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* For Part IX see This Bulletin, **24**, 598 (1960).

[1] Y.R. Naves, *Helv. Chim. Acta*, **25**, 732 (1942).

[2] Y.R. Naves and G. Papazian, *Helv. Chim. Acta*, **25**, 1023 (1942).

[3] Glidden Co., Brit. P. 755, 667, *C.A.*, **51**, 8797 (1957).

[4] E.D. Bergmann and P. Bracha, *J. Org. Chem.*, **24**, 994 (1959).

[5] A.R. Penfold, G.R. Ramage and J.L. Simonsen *J. Chem. Soc.*, **1939**, 1479.

[6] C. Blant, C.A. Vodoz, H. Kappeler and H. Schinz, *Helv. Chim. Acta*, **34**, 724 (1951).

[7] W. Kuhn and H. Schinz, *Helv. Chim. Acta*, **36**, 163 (1953).

[8] T. Suga and T. Matsuura, *Bull. Chem. Soc. Japan*, **31**, 576 (1958).

[9] S. Shimizu, H. Ueda and N. Ikeda, This Bulletin, **24**, 323 (1960).

[10] H. Ueda and S. Shimizu, This Bulletin, **23**, 524 (1959).

[11] H. Ueda and S. Shimizu, This Bulletin, **24**, 92 (1960).

[12] H. Ueda, This Bulletin, **24**, 598 (1960).

[13] A.K. Macbeth, *J. Chem. Soc.*, **1951**, 1848; **1952**, 2852, 4748; **1953**, 901.

isomenthyl 3,5-dinitrobenzoate were isolated pure from the reduction product. On the other hand, oxidation of the reduction product gave menthone mixture which was identified by infrared analysis.

On the basis of these data, it seemed reasonable to assume that Bergmann's piperitenone might be composed of cross conjugated C_{10} ketone at least in part, if not piperitenone previously reported. It is of mechanistic interest to see how cross conjugated ketone is reduced to a saturated alcohol on the reduction with sodium in aqueous ammonia. Its mechanism is worthy of investigation.

Since methylvinylketone can be easily prepared by the condensation of acetone with formaldehyde, and mesityloxide from acetone, the result of this investigation suggests the possibility of commercial production of menthol from acetone and formaldehyde.

EXPERIMENTAL

All melting and boiling points were uncorrected. Ultra violet spectra were determined at Prof. Shimizu's Laboratory, Shinshu University. Microanalysis was carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto.

Bergmann's Piperitenone: According to the procedure of Bergmann and Bracha[4], the ketone was prepared from the condensation product of mesityloxide with methylvinylketone[14]; b.p. 96～98°/7 mm., d_4^{25} 0.9682 (Naves[2],[a] reports d_4^{20} 0.9774), n_D^{25} 1.5211 (Naves reports n_D^{20} 1.5294), λ_{max} 238, 296 mμ in methanol (Naves reports 242, 278 mμ; Bergmann reports 238, 295.5 mμ), 2,4-dinitrophenylhydrazone m.p. 157° (Naves reports 184°; Bergmann reports 152°)

Reduction of Bergmann's Piperitenone: Bergmann's piperitenone (8.32 g) was reduced with sodium (8.0 g) in aqueous ammonia and benzene (reaction temp. 0～5°), in the same way as previously reported[15]. The product (Product-I) was a colorless oil with a characteristic odour. No crystal could be isolated from Product-I on cooling (−22°); b.p. 75～77°/4.5 mm., d_4^{22} 0.9019, n_D^{22} 1.4683, Yield 6.48 g. Infrared absorption spectrum showed that Product-I contained mainly alcohols, though very weak absorptions at 240 and 264 mμ in the ultraviolet region indicated that a little carbonyl compound was still present.

Product-I resists catalytic hydrogenation. This substance (3.05 g) was dissolved in ethanol (15 ml) and hydrogenated over platinum oxide (40 mg). It absorbed not more than 26 ml (17°) of hydrogen during two hours, equivalent to 5.5% of the theory on the basis of pulegol or piperitol. Then, it was further reduced in ethanol containing a Raney nickel catalyst at 145° under 102 Kg/cm² for 1.5 hours. The product (Product-II) was a colorless oil possessing an odour very similar to that of Product-I; b.p. 89～91°/6 mm, d_4^{20} 0.8859, n_D^{20} 1.4638, Yield 2.53 g. Infrared spectrum of Product-II agreed in every respect with that of Product-I.

From this fact, it was found that Product-I was a saturated alcohol, which was identified to be composed mainly of (±)-menthol and (±)-isomenthol by comparison of its infrared spectrum with those of the authentic samples[16]. Absorption bands, 3340, 2950, 1460, 1372, 1354, 1299, 1180, 1080, 1044, 1026, 994, 875 cm^{-1} were found commonly in both menthol and isomenthol, 1260, 1218, 1101, 977, 918, 845 cm^{-1} characteristic of menthol, and 1150, 1133, 967, 948 cm^{-1} characteristic of isomenthol.

Separation of Menthol Isomers: Product-I (1.41 g) gave crude 3,5-dinitrobenzoate (1.26 g), m.p. 100～103.5°, after one crystallisation from *n*-hexane. No crystalline ester could be isolated from the resulting mother liqor, but dehydration of ester was not observed[b]. Further two crystallisations of the crude ester (523 mg) from methanol gave a homogeneous fraction (361 mg) as colorless needles, m.p. 120°, unaltered by further crystallisations. Mixed melting points with (±)-menthyl, (±)-isomenthyl and (±)-neomenthyl 3,5-dinitrobenzoates were 122～123°, 120° and 107～115° respectively. *Anal.* Found: C, 58.37; H, 6.40; Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.27; H, 6.33%.

The chromatography of this 3,5-dinitrobenzoate of m.p. 120° (30.0 mg) on alumina column (Brockmann Alumina 9.0 g, column diameter 11 mm, elution solvent *n*-hexane) gave two fractions, (±)-menthyl and (±)-isomenthyl 3,5-dinitrobenzoate, as shown in Table I. Both fractions were identified by mixed melting

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[14] A. Wohl and A. Prill, *Ann.*, 139 (1924).

[a] Naves' report is of natural piperitenone.

[15] H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin, **23**, 376 (1959).

[16] H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

[b] If the Product-I was composed of pulegol, this ester would be dehydrated to unsaturated hydrocarbons and 3,5-dinitrobenzoic acid, as reported by Nacbeth.

TABLE I. ABSORPTION CHROMATOGRAPHY OF 3,5-DINITROBENZOATE OF m.p. 120°

Fraction No.	Elution Solvent Total	Recovery Material Weight	Recovery Material Melting point	
	ml	mg	°C	
1~8	80	0	—	
9	90	2.3	128	(±)-Menthyl 3,5-dinitrobenzoate
10	100	2.9	128	
11	110	3.2	128	
12	120	2.8	127.5	
13	130	2.3	121.5	
14	140	2.0	120	
15	150	1.5	118.5	Mixture
16	160	1.6	117	
17	170	1.3	114	
18	180	1.3	116	
19	190	0.8	125	
20	200	1.3	127	
21	210	1.1	127.5	
22	220	0.8	127.5	(±)-Isomenthyl 3,5-dinitrobenzoate
23	230	0.4	128	
24	240	0.3	128.5	
25	250	0.2	129	
26*	260	2.8	128.5	

Total 28.9 mg (Recovery 96%)

* Fraction 26 was eluted by ether.

points and infrared spectra in comparison with authentic samples[17].

Esterification of Product-II also gave the same 3,5-dinitrobenzoate, m.p. 120°, as prepared from Product-I, and mixed melting point was not depressed.

Oxidation of Reduction product of Bergmann's Piperitenone: The oxidation of Product-I (1.55 g) with sodium dichromate in acetic acid at 32~33° gave a colorless oil (1.00 g); b.p. 63~65°/5 mm., d_4^{18} 0.8976 n_D^{18} 1.4645. Although a very weak absorption at 233 mμ in the ultraviolet spectrum indicated that a little unsaturated carbonyl compound was present, infrared spectrum showed that the oxidation product was composed mainly of (±)-menthone and (±)-isomenthone. Absorption banks, 2950, 1720, 1457, 1370, 1331, 1310, 1248, 1130 cm^{-1} were found commonly in both menthone and isomenthone, 1117, 1092, 1048, 991, 889, 838 cm^{-1} characterislic of menthone, and 1273, 1230, 1204, 1080, 1030, 969 cm^{-1} characteristic of isomenthone. No absorption band responsible for pulegone or piperitone was shown in this spectrum.

Acknowledgements The author is indebted to Emeritus Prof. S. Takei, Prof. T. Mitsui and Prof. M. Nakajima, University of Kyoto, for their kind advices and suggestions. He also wishes to express his sincere thanks to Prof. S. Shimizu, Shinshu University, and Dr. Y. Inouye, Institute for Chemical Research, University of Kyoto, for their kind guidances throughout this series, and to Mr. K. Fujimoto for his assistance in carrying out this work.

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[17] H. Ueda, This Bulletin, **24**, 506 (1960).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 604~609, 1960]

Polarographic Studies on Sarkomycin

Part III. Polarography of Isoniazid and Sarkomycin-isoniazid Derivative. 1.

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By polarography, isoniazid (INH) gives three reduction waves and sarkomycin-isoniazid derivative (SK-INH) gives three or four waves. Polarography of the mixtures of two out of sarkomycin (SK), INH and SK-INH suggests that the first, the second and the fourth wave of SK-INH are derived from the first, the second and the third wave of INH, respectively, and the third wave from SK. Alternating current polarography of SK-INH shows three or four maxima, whose numbers are the same as wave numbers in direct current polarography.

Hara et al.[1,2] reported on sarkomycin-isoniazid derivative (SK-INH) to have an antitumor activity and a stability superior to sarkomycin (SK). Though its structure is not well known, SK-INH gave interesting polarographic results, compared with isoniazid (INH) and SK[3], components of SK-INH.

As Tachi et al.[4] reported, INH gives three polarographic waves; the first and the second wave are close to each other and the third wave is observed just before the hydrogen wave appears. Half-wave potentials of these waves become less positive with increasing pH. Effect of pH on half-wave potential is given in Table I and the polarograms are shown in Fig. 1. The plot of half-wave potential *vs.* pH yields straight lines with slopes ($\Delta E/\Delta pH$) of 0.98, 0.90 and 0.54 for the first, the second and the third wave, respectively. Limiting currents, obtained at different mercury head in citrate buffer of pH 7.0 reveal that they are not kinetic as shown in Table II. Assuming that the reductions proceed reversibly, the waves of INH

TABLE I. HALF-WAVE POTENTIALS OF INH AT VARIOUS pH VALUES

pH	Half-wave potential vs. S.C.E. I	 II	 III
2.5	−0.61V	−0.76V	−1.31V
3.3	−0.68	−0.83	−1.36
4.3	−0.77	−0.92	−1.42
5.4	−0.90	−1.04	−1.47
6.2	−0.98	−1.09	−1.52
7.1	−1.06	−1.16	−1.56
8.3	−1.16	−1.28	−1.62

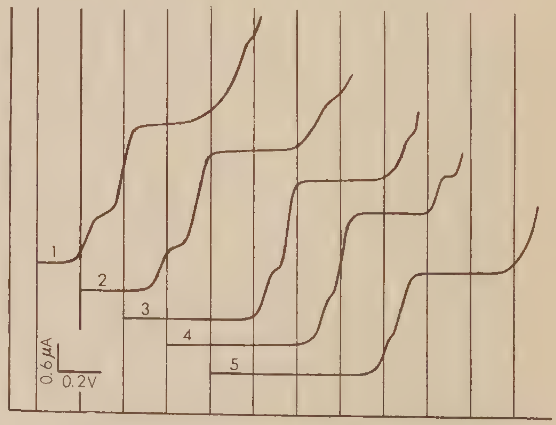

FIG. 1. Polarograms of INH from −0.4 V vs. S.C.E.
1. pH 2.5; 2. pH 3.3; 3. pH 4.3; 4. pH 7.1; 5. pH 8.3.

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1) T. Hara, Y. Yamada, E. Akita and H. Goi, *J. Antibiotics (Japan)*, Ser. B, **9**, 221~6 (1956).

2) T. Hara, Y. Yamada and E. Akita, *J. Antibiotics (Japan)*, Ser. A, **10**, 62~5 (1957).

3) K. Ida, This Bulletin, **23**, 40~5 (1959).

4) I. Tachi and Y. Nagata, *Kagaku no Ryoiki*, **6**, 490~1 (1952).

TABLE II. EFFECT OF MERCURY HEAD (h) ON DIFFUSION CURRENT (I_d) OF INH IN CITRATE BUFFER OF pH 7.0.

h	1st wave		2nd wave		3rd wave	
	I_d	$I_d/h^{\frac{1}{2}}$	I_d	$I_d/h^{\frac{1}{2}}$	I_d	$I_d/h^{\frac{1}{2}}$
cm	μA		μA		μA	
100	0.86	0.09	1.48	0.15	0.90	0.09
81	0.81	0.09	1.38	0.15	0.96	0.11
64	0.68	0.08	1.15	0.14	1.03	0.13

TABLE III. HALF-WAVE POTENTIALS OF SK-INH AT VARIOUS pH VALUES

pH	Half-wave potential vs. S.C.E.		
	I	II	III
2.5	−0.65V	−0.77V	−0.93V
3.4	−0.71	−0.84	−0.98
4.4	−0.78	−0.93	−1.08
5.4	−0.88	−1.02	−1.18
6.3	−0.97	−1.10	−1.25
7.0	−1.05	−1.17	−1.32
7.9	−1.14	−1.25	−1.40

obtained were analyzed; Values of ΔE against $\log i/(i_d-i)$ were 0.034 V, 0.030 V and 0.056 V for the first, the second and the third wave, respectively, which gave electron numbers of 1.74, 1.97 and 1.05 for respective wave by calculation.

On the other hand, polarography of SK-INH resulted in three or four waves. Half-wave potentials at various pH values are given in Table III and the polarograms in Fig. 2. The first wave is close to the second wave and the limiting current of the third wave is much less than those of the first and the second wave. The fourth wave appears just before the hydrogen wave appears, but it is not recognized in some cases. The reason why the fourth wave does not appear in some cases is not clear and should be investigated later. Half-wave potential shows a linear relationshop with pH and the slopes have values of 0.92, 0.90 and 0.88 V for the first, the second and the third wave, respectively. Effect of mercury head on limiting currents in citrate buffer of pH 7.0 proves that they are not kinetic as shown in Table IV. Assuming that the reductions proceed reversibly, values of ΔE *vs.* $\log i/(i_d-i)$ calculated from the first, the second, the third and the fourth wave are 0.035 V, 0.027 V, 0.027 V and 0.038 V and the electron number concerned is 1.69, 2.19, 2.19 and 1.55, respectively.

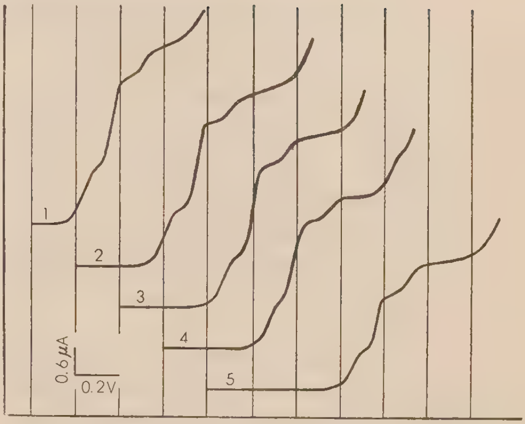

FIG. 2. Polarograms of SK-INH from −0.5 V vs. S.C.E.
1. pH 3.3; 2. pH 5.4; 3. pH 6.2; 4. pH 7.1; 5. pH 8.3.

TABLE IV. EFFECT OF MERCURY HEAD (h) ON DIFFUSION CURRENT (I_d) OF SK-INH IN CITRATE BUFFER OF pH 7.0.

h	1st wave		2nd wave		3rd wave	
	I_d	$I_d/h^{\frac{1}{2}}$	I_d	$I_d/h^{\frac{1}{2}}$	I_d	$I_d/h^{\frac{1}{2}}$
cm	μA		μA		μA	
81	8.5	0.94	10.4	1.16	4.1	0.46
64	7.6	0.95	9.3	1.16	3.6	0.45
49	6.7	0.96	8.0	1.14	3.1	0.44

A mixture of SK and INH was polarographed to give four waves in citrate buffer of pH 1.8, as given in Table V and Fig. 3, along with the results by SK and INH alone. The half-wave potentials of the reduction waves of the mixture are similar to those of SK-INH. Moreover, the half-wave potentials and the diffusion currents of the first and the second wave of the mixture are similar to those of INH only, comparing these waves with those from the same amount of INH as the added. Besides, the mixture gives a similar half-wave potential to that of the third wave of INH, even though the current is different. Comparing the waves by a mixture of SK and INH with those of SK equivalent to the amount used in the mixture, half-wave potential of the third wave in the

Table V. Diffusion Current of SK, INH and Their Mixture in Citric Acid Aqueous Solution (pH 1.8).

	Concn.	Half-wave potential vs. S.C.E.				
		−0.68V	−0.77V	−0.90V	−1.07V	−1.13V
SK	1.8 u./ml	—	—	1.03μA	1.26μA	—
INH	20 γ/ml	0.79μA	1.07μA	—	—	0.53μA
SK+INH	SK 1.8 u./ml INH 20 γ/ml	0.78	0.95	0.74	—	0.95

Table VI. Diffusion Current of SK, SK-INH and Their Mixture in Citric Acid Aqueous Solution (pH 1.8).

	Concn.	Half-wave potential vs. S.C.E.				
		−0.68V	−0.77V	−0.90V	−1.07V	−1.13V
SK	1.8 u./ml	—	—	1.03μA	1.26μA	—
SK-INH	34 γ/ml	0.47μA	0.24μA	0.18	—	0.38μA
SK+SK-INH	SK 1.8 u./ml SK-INH 34 γ/ml	0.42	0.36	0.95	Ill-defined	Ill-defined

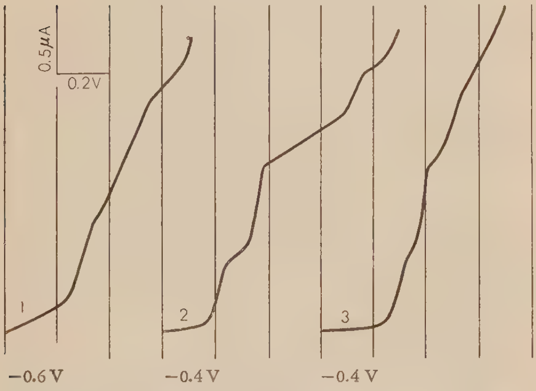

Fig. 3. Polarograms of SK (1), INH (2) and Their Mixture (3) in Citric Acid Aqueous Solution (pH 1.8)

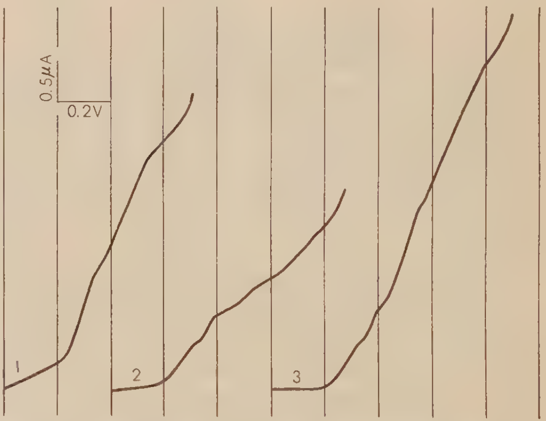

Fig. 4. Polarograms of SK (1), SK-INH (2), and Their Mixture (3) in Citric Acid Aqueous Solution (pH 1.8) from −0.4 V vs. S.C.E.

mixture is similar to that of the first wave of SK, but the former diffusion current is slightly less than the latter one. The most significant difference exists in the fact that the mixture gives no wave at the potential where the second wave of SK is observed.

A mixture of SK and SK-INH was polarographed in citrate buffer of pH 1.8 and the results are given in Table VI and Fig. 4, together with the results of both SK and SK-INH both the mixture and SK-INH give similar half-wave potentials of the first and the second wave, but the former diffusion current is slightly larger than the latter one. Moreover, the third wave of the mixture is observed at the potential where the third wave of SK appears. The waves equivalent to both the second wave of SK and the fourth wave of SK-INH appear to coalesce and no exact values of half-wave potential and no diffusion currents can be measured. However, they seem to have similar values to SK or SK-INH.

The results that the above experiments give

TABLE VII. DIFFUSION CURRENT OF SK, INH, SK-INH AND THEIR MIXTURE IN CITRATE BUFFER (pH 7.0).

	Concn.	Half-wave potential vs. S.C.E.			
		−1.05V	−1.15V	−1.30V	−1.56V
SK	1.8 u./ml	—	—	1.37μA	—
INH	20 γ/ml	0.81μA	1.20μA	—	1.12μA
SK-INH	34 γ/ml	0.25	0.23	0.09	—
SK+INH	SK 1.8 u./ml INH 20 γ/ml	0.71	1.15	1.28	0.93
SK+SK-INH	SK 1.8 u./ml SK-INH 34 γ/ml	0.37	0.38	1.50	—

TABLE VIII. DIFFUSION CURRENT OF SK-INH, INH AND THEIR MIXTURE IN CITRATE BUFFER (pH 7.0).

	Concn.	Half-wave potential vs. S.C.E.			
		−1.05V	−1.15V	−1.30V	−1.56V
SK-INH	52.1 γ/ml	0.52μA	0.43μA	0.15μA	0.15μA
INH	13.3 γ/ml	0.49	0.87	—	0.73
SK-INH+INH	SK-INH 52.1 γ/ml INH 13.3 γ/ml	0.94	1.29	0.11	0.96

TABLE IX. DIFFUSION CURRENT OF SK-INH, INH AND THEIR MIXTURE IN CITRIC ACID AQUEOUS SOLUTION (pH 1.8).

	Concn.	Half-wave potential vs. S.C.E.			
		−0.68V	−0.77V	−0.90V	−1.13V
SK-INH	34 γ/ml	0.47μA	0.24μA	0.18μA	0.38μA
INH	20 γ/ml	0.79	1.07	—	0.53
SK-INH+INH	SK-INH 34 γ/ml INH 20 γ/ml	1.21	1.30	0.20	0.88

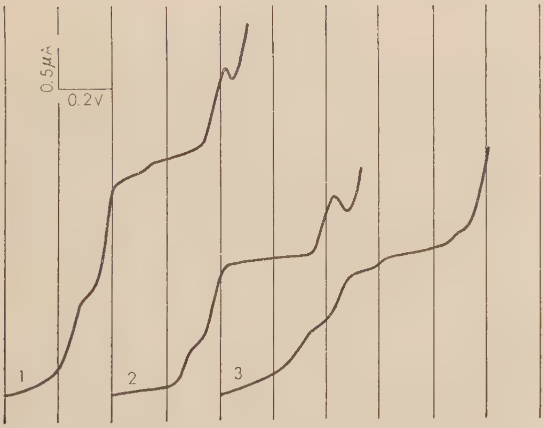

FIG. 5. Polarograms of SK-INH (3), INH (2), and Their Mixture (1) in Citrate Buffer (pH 7.0) from −0.8 V vs. S.C.E.

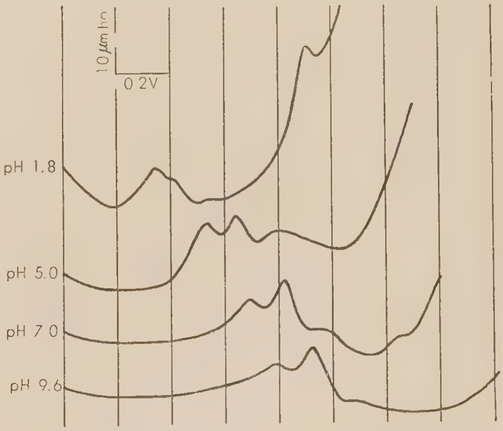

FIG. 6. A.C. Polarograms of SK-INH from −0.5 V vs. S.C.E.

Table X. Summit Potentials of SK-INH at Various pH Values by A.C. Polarography

pH	Summit potential vs. S.C.E.			
	I	II	III	IV
1.8	−0.75V	−0.82V	−0.95V	−1.30V
3.0	−0.80	−0.87	−0.99	−1.36
4.0	−0.86	−0.95	−1.09	−1.45
5.0	−0.94	−1.05	−1.20	
6.0	−1.00	−1.12	−1.28	
7.0	−1.09	−1.22	−1.36	−1.64
8.0	−1.15	−1.28	−1.43	−1.71
9.6	−1.19	−1.32	−1.47	

Table XI. Analysis of Polarographic waves of SK-INH and INH in Citric Acid Aqueous Solution (pH 1.8).

$E_{\frac{1}{2}}$		SK-INH (34 γ/ml)	INH (20 γ/ml)
−0.68V	I_d	0.47 μA	0.79 μA
	$\Delta E/\log i/(i_d-i)$	0.035 V	0.034 V
	$\Delta E/\Delta \mathrm{pH}$	0.92 V	0.98 V
−0.77V	I_d	0.24 μA	1.07 μA
	$\Delta E/\log i/(i_d-i)$	0.027 V	0.030 V
	$\Delta E/\Delta \mathrm{pH}$	0.90 V	0.90 V
−1.13V	I_d	0.38 μA	0.53 μA
	$\Delta E/\log i/(i_d-i)$	0.038 V	0.056 V
	$\Delta E/\Delta \mathrm{pH}$	0.88 V	0.54 V

in citrate buffer of pH 7.0 are similar to the above described as shown in Table VII.

Polarograms and their data of SK-INH, INH and their mixture are given in Fig. 5 and Table VIII and IX. The first, the second and the fourth wave of the mixture have similar half-wave potentials to the first, the second and the third wave of INH, respectively, and the former diffusion currents are almost equal to the sum of the diffusion currents of the corresponding waves by each component. The wave, corresponding to the third wave of SK-INH, does not appear in the mixture, so the observed diffusion current is equal to that of the added SK-INH.

Fig. 6 shows alternating current polarograms of SK-INH and the data are listed in Table X. SK-INH gives three or four maxima by alternating current polarography, but the fourth wave is not observed at some pH values. The numbers of the maxima in alternating current polarography are the same as those of the waves in direct current polarography, but the former summit potentials are slightly different from the latter half-wave potentials. The most distinct difference between alternating and direct current polarography exists in the inclination of the pH-summit as well as half-wave potential curve of the first wave; the value of $\Delta E/\Delta \mathrm{pH}$ was 0.92 by direct current polarography and 0.72 by alternating current polarography.

Comparison of half-wave potentials of SK-INH and INH is summarized in Table XI. Their first and the second waves have similarities as follows:

1. Their half-wave potentials coincide with one another within error.
2. Both pH-half-wave potential curves have similar slopes.
3. Both polarographic waves have similar slopes.

Polarography of mixtures which were comprised of the two out of SK, INH and SK-INH, resulted in the supposition that the first and the second wave of SK-INH would be derived from the first and the second wave of INH and the third wave of SK-INH from the first wave of SK. The facts that SK-INH and a mixture of SK and INH give no wave corresponding to the second wave of SK but a mixture of SK and SK-INH gives it, will help to make clear the structure of SK-INH.

EXPERIMENTALS

Apparatus: A 'Yanagimoto' Model AP-20 Potential Scanner for polarograph with a mirror galvanometer (sensitivity, 1.86×10^{-3} μA/mm/m; critically damped period, 5 seconds; internal resistance, 850 ohms; critical damping resistance, 5000 ohms) was used. Alternating current polarography was carried out by using a 'Yanagimoto' Model PA-101 Polarograph. The used cell was a H-type cell with an agar bridge to a saturated calomel electrode to determine the half-wave potentials. The dropping mercury electrode had a value of $m^{2/3}\,t^{1/6}$ of 2.52 mg$^{2/3}$ sec$^{1/6}$. The temperature was maintained constant at 21°C during electrolysis.

Samples: SK-INH. SK-INH was prepared as described by Hara et al.[1] On hundred g of SK acid oil was dissolved in 200 ml of isopropanol. After gradual addition of 10 g of INH suspended in isopropanol, the mixture was left stand for 3 hours at 40°C. The solution, colored deep red, was filtered and the filtrate was chilled at $-20°C$ overnight. The jelled solution was filtered cooling. Rinsed with cold isopropanol and ether, the residue was dried *in vacuo* at room temperature. Re-purification from isopropanol yielded about 30 g of SK-INH. The biological activity was 1 unit/mg using SK as a standard.

INH. Crystal of Nipren (Tanabe Pharmaceutical Co.) was used.

Citrate buffer. About 0.1 M aqueous solution of citric acid and about 0.2 M aqueous solution of disodium phosphate were mixed to be measured the pH with a glass electrode pH meter.

Polarography of INH: Ten mg of INH was dissolved in 50 ml of water. One ml of the resulting solution was diluted to 10 ml with citrate buffer and polarographed after removal of dissolved oxygen.

Polarography of SK-INH: Ten mg of SK-INH was dissolved in 25 ml of water. One ml of the resulting solution was diluted to 10 ml with citrate buffer and polarographed after removal of dissolved oxygen.

Polarography of a mixture of SK and INH: Half a ml of aqueous solution of SK (36 units/ml) was added to 0.6 ml of INH aqueous solution (33.3 mcg/ml) and made up to 10 ml with citrate buffer of pH 1.8 or 7.0 to be polarographed after removal of dissolved oxygen. Both the SK solution and INH solution were similarly polarographed after the same dilution.

Polarography of a mixture of SK and SK-INH: Half a ml of SK aqueous solution (36 units/ml) and 1 ml of an aqueous solution of SK-INH (340 mcg/ml) were mixed and made up to 10 ml with citrate buffer of pH 1.8 or 7.0 to be polarographed after removal of dissolved oxygen. Both the SK solution and SK-INH solution were similarly polarographed after the same dilution.

Polarography of a mixture of SK-INH and INH: One ml of an aqueous solution of SK-INH (521 mcg/ml) and 0.4 ml of an aqueous solution of INH (332.9 mcg/ml) were mixed and made up to 10 ml with citrate buffer of pH 1.8 or 7.0 to be polarographed after removal of dissolved oxygen. Both the SK-INH solution and INH solution were similarly polarographed after the same dilution.

Alternating current polarography of SK-INH: Two ml of SK-INH aqueous solution (340 mcg/ml) was diluted to 10 ml with citrate buffer and polarographed after removal of dissolved oxygen.

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Polarographic Studies on Sarkomycin

Part IV. Polarography of Isoniazid and Sarkomycin-isoniazid Derivative. 2.

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Quantitative analyses of sarkomycin-isoniazid derivative (SK-INH), isoniazid (INH) and their mixture are discussed. The diffusion current of SK-INH gives a linear relationship with the concentration only in the same lot, but this calibration curve cannot be applied for samples of other lots. Increase in the diffusion currents of the first and the second wave in a mixture of SK-INH and INH yields straight lines against the added INH, which coincide well with the calibration curve of INH. Relation between diffusion currents of the first and the second wave and nitrogen contents, obtained by a lot of SK-INH, cannot be used for other lots of sample. Ratios of the diffusion currents of these waves show different values in each sample, which are larger than in INH.

Polarographic behavior of sarkomycin-isoniazid derivative (SK-INH) were previously reported to be similar to those of isoniazid (INH)[1]: The first, the second and the fourth wave of SK-INH gave similar half-wave potentials to those of the first, the second and the third wave of INH, respectively. Besides, their pH-half-wave potential curves and reduction waves showed similar values of slopes. Results obtained by a mixture of sarkomycin (SK) and INH suggested that the first and the second wave of SK-INH was caused by the first and the second wave of INH, a component of SK-INH.

At any pH, INH gives well-defined waves applicable for quantitative analysis. Diffusion currents of the first, the second and the third wave are related linearly with concentrations as given in Fig. 1 and 2, and their currents are stable within 24 hours as in Table I. As for the third wave, a linear calibration curve can be written in low concentration, but no proportionality is observed in high concentrations.

The first and the second wave of SK-INH are well-defined and suitable for analytical application. The third wave is not adequate for analysis, because its diffusion current is much less than those of the other waves. However,

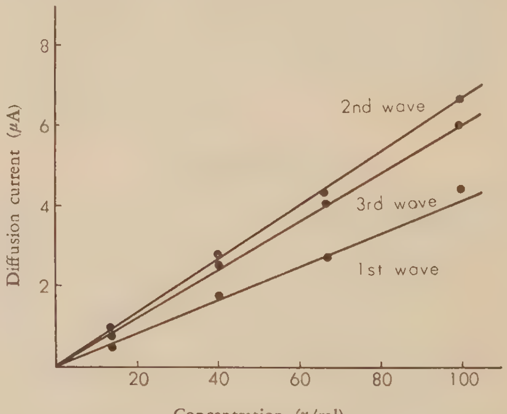

FIG. 1. Calibration Curves of INH in Citrate Buffer (pH 7.0).

TABLE I. STABILITY OF DIFFUSION CURRENT OF INH IN CITRATE BUFFER (pH 7.0).

Hrs.	1st wave	2nd wave	3rd wave
0	100.0%	100.0%	100.0%
4	99.9	100.0	100.0
24	100.0	98.7	96.2

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[1] K. Ida, This Bulletin, **24**, 604~9 (1960).

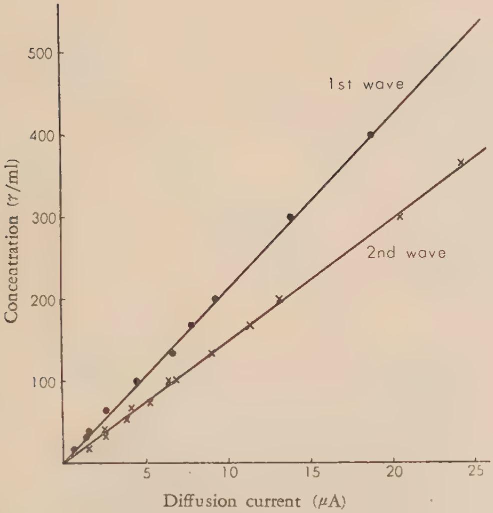

FIG. 2. Calibration Curves of INH in Citrate Buffer (pH 7.0).

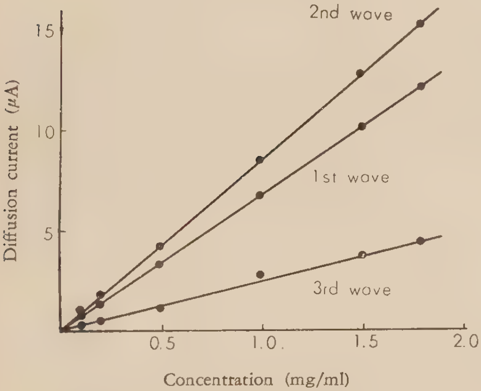

FIG. 3. Calibration Curves of SK-INH in Citrate Buffer (pH 7.0).

all waves have straight line between diffusion currents and concentrations, as shown in Fig. 3.

TABLE II. STABILITY OF DIFFUSION CURRENT OF SK-INH IN CITRATE BUFFER (pH 7.0).

Hrs.	1st wave	2nd wave	3rd wave
0	100.0%	100.0%	100.0%
4	99.0	100.0	100.0
24	94.9	95.0	100.0

TABLE III. DIFFUSION CURRENTS OF SK-INH PER UNIT OF BIOLOGICAL ACTIVITY

Sample	1st wave	2nd wave	3rd wave
1	7.0μA/unit/ml	20.0μA/unit/ml	6.0μA/unit/ml
2	8.3	14.8	4.2
3	30.5	43.5	15.5
4	27.0	29.5	8.0
5	150.0	122.5	41.7
6	29.0	47.0	87.0
7	0.2	0.4	0.1
7*	13.0	22.6	7.3
8	0.2	0.3	0.1
8*	12.0	18.7	6.4

* Determined after standing in aqueous solution for 14 days.

TABLE IV. INFLUENCE OF TIME ON DIFFUSION CURRENT AND BIOLOGICAL ACTIVITY OF SK-INH.

All samples were polarographed after addition of 4.9 ml of citrate buffer of pH 7.0 to 0.1 ml of sample solution.

Sample	Potency	1st wave	2nd wave	3rd wave
7	14.8 unit/ml	3.42 μA	3.81 μA	1.53 μA
7*	0.2	2.40	3.73	1.28
8	19.6	3.82	8.26	1.96
8*	0.3	3.90	6.77	2.18

* Determined after standing in aqueous solution for 2 weeks.

SK-INH in aqueous solution of pH 7.0 lost no antibacterial activity for two days. On the other hand, SK-INH in citrate buffer of pH 7.0 showed no decrease in diffusion current after four hours as in Table II. But samples from different lots and samples whose antibacterial activities decreased by letting stand for two weeks in aqueous solution gave significantly different values from one another as given in Table III. Samples left for two weeks in aqueous solution gave 50~70 times larger diffusion current per biological unit than the original samples. The detail is given in Table IV and the diffusion currents were not decreased so much as the biological activities. These decrease was found not only in the first and the second wave, probably caused by INH, but also in the third wave, probably caused by SK.

Because SK-INH is comprised of SK (2-methylene-3-oxocyclopentane-1-carboxylic acid) and INH[2], the containing nitrogen is derived from INH. Therefore, INH content in SK-INH can be represented by nitrogen content. Regressions of nitrogen content upon diffusion current of the first and the second wave, calculated by four lots of SK-INH with different nitrogen content, are listed in Table V. These regressions were statistically different. These

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[2] T. Hara, Y. Yamada and E. Akita, *J. Antibiotics (Japan)*, Ser. A, **10**, 62~5 (1957).

TABLE V. REGRESSIONS OF NITROGEN CONTENT OF SK-INH AND INH UPON DIFFUSION CURRENT IN CITRATE BUFFER OF pH 7.0.

Sample	1st wave	2nd wave
A	$y=14.48x-3.54$	$y=10.07x-0.85$
B	$y=11.30x-0.65$	$y=8.42x-0.70$
C	$y=14.99x-2.40$	$y=12.06x-1.28$
D	$y=10.59x-1.27$	$y=13.57x-2.97$
INH	$y=6.45x+0.95$	$y=4.56x+0.09$

x: nitrogen content (γ/ml), y: diffusion current (μA)

TABLE VI. ANALYSIS OF SK-INH SAMPLES

Sample	Elemental analysis			Approx. value of INH/SK by wt.
	C	H	N	
A	57.46%	5.88%	7.97%	0.35
B	57.77	6.11	7.21	0.31
C	57.90	5.92	6.90	0.29
D	56.74	5.67	14.06	0.85

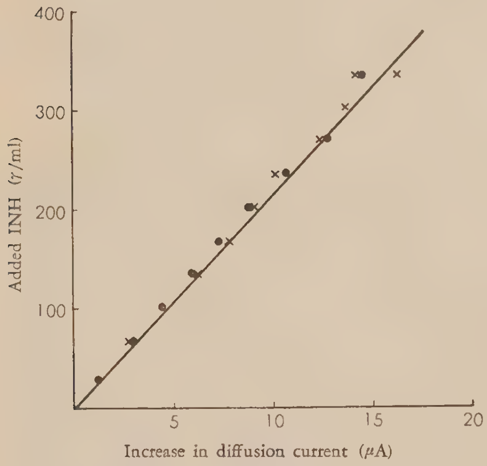

FIG. 4. Correlation between Added INH and Increase in Diffusion Current of the First Wave of SK-INH in Citrate Buffer (pH 7.0).
× Sample A, • Sample B.

results show that the diffusion current of the first and the second wave gives no linear relationship with nitrogen content of all samples and nitrogen content can not be determined polarographically.

The diffusion currents by the first and the second wave of a mixture of SK-INH and INH are equal to the sum of those from each component, as previously reported. The amount of the added INH was varied to ascertain this fact. Furthermore, this fact was investigated by samples with different ratio of INH to SK. The samples used here are shown in Table VI and the nitrogen content is ranged from 6.9 to 14.1%. The increase in diffusion currents of the first and the second wave, which were obtained by adding various amount of INH to

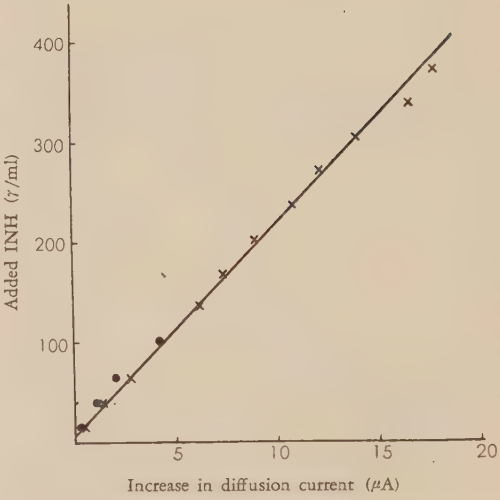

Fig. 5. Correlation between Added INH and Increase in Diffusion Current of the First Wave of SK-INH in Citrate Buffer (pH 7.0).
× Sample C, • Sample D.

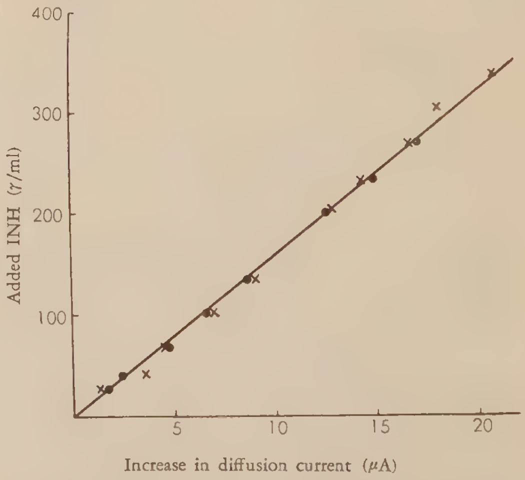

FIG. 6. Correlation between Added INH and Increase in Diffusion Current of the Second Wave of SK-INH in Citrate Buffer (pH 7.0).
• Sample A, × Sample B.

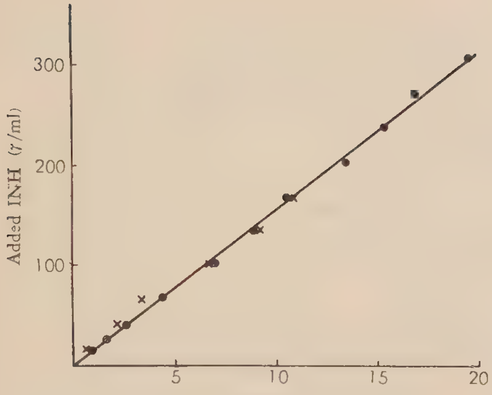

FIG. 7. Correlation between Added INH and Increase in Diffusion Current of the Second Wave of SK-INH in Citrate Buffer (pH 7.0).
• Sample C, × Sample D.

a fixed volume of aqueous solution of these samples, is shown in Fig. 4~7. Variance of nitrogen content has no influence on the value and gives constant values according to the amount of the added INH. Moreover, the slopes of the increase in diffusion current *vs.* the amount of the added INH coincide well with the calibration curve of INH.

Ratio of the current of the first wave to that of the second wave at pH 7.0 is 0.82, 0.76, 0.90 and 1.13 for sample A, B, C and D, respectively, but the ratio for INH is 0.52, which is different from them. Though the diffusion currents equivalent to the added INH are well recovered from a mixture of SK and INH and a mixture of SK-INH and INH, INH as a component of SK-INH is not recorved so well as from the mixtures. If INH contained in SK-INH would be dissociated in an aqueous solution, the polarographic behavior would not be so different from free INH or INH in the mixture. It is very interesting that SK-INH gives the same half-wave potentials as INH but quantitatively different behaviors.

The fourth wave of SK-INH gives a proportionality between the diffusion current and the low concentration, but no linear relationship in high concentration, and a constant value is attained eventually. In much higher concentration it is not observed. This wave should be further studied.

EXPERIMENTAL

Apparatus: A "Yanagimoto" Model AP-20 Potential Scanner for polarograph with a mirror galvanometer (sensitivity, 1.86×10^{-3} μA/mm/m; critically damped period, 5 seconds; internal resistance, 850 ohms; critical damping resistance, 5000 ohms) was used. The dropping mercury electrode had a value of $m^{2/3}\ t^{1/6}$ of 2.38 $mg^{2/3}\ sec^{1/6}$. The temperature was maintained constant at 15°C during electrolysis.

Samples: SK-INH. The used samples were the ones prepared by Mr. Hara and the ones prepared by mass production at Kawasaki Plant of Meiji Seika Kaisha.

INH. Crystal of Nipren (Tanabe Pharmaceutical Co.) was used.

Citrate buffer. About 0.1 M citric acid and about 0.2 M disodium phosphate was mixed and adjusted to pH 7.0.

Polarographic determination of INH: Aqueous solution of sample (0.1~1 ml) was diluted to 10 ml with citrate buffer and polarographed after removal of dissolved oxygen. INH was determined by comparing the obtained diffusion current of the first or the second wave with a standard curve, prepared by the same procedure using known amounts of INH.

Polarography of SK-INH: Aqueous solution of sample (0.1~1 ml) was diluted to 10 ml with citrate buffer and polarographed after removal of dissolved oxygen. The diffusion current of the first, the second, the third or the fourth wave was measured.

Polarography of a mixture of SK-INH and INH: Half a ml of aqueous solution of SK-INH was mixed with 0.5 ml of aqueous solution containing various amount of INH and diluted to 10 ml with citrate buffer. The solution was polarographed after removal of dissolved oxygen and the diffusion currents of the first and the second wave were measured. Half a ml of the SK-INH solution was diluted to 10 ml with citrate buffer and the diffusion currents of the first and the second wave were similarly measured. Difference between the diffusion currents by the mixture and those by SK-INH was considered as the increase amount.

Acknowledgements The author wishes to acknowledge kind guidance and valuable suggestions extended by Dr. Isamu Tachi, Professor of Kyoto University, and thank Mr. Takeshi Hara, Chief Chemist of the Research Laboratory of Meiji Seika Kaisha, Ltd., for giving the samples and Mr. Shozo Kokubo, Manager of Yanagimoto Co., for supplying the apparatus.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 614~617, 1960]

Blätteralkohol (VIII)[†]:

Über das Vorkommen des *trans*-Isomeren in der natürlichen Blätteralkohol-fraktion[††]

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Institut für Chemische Forschung, Universität Kyoto

Eingegangen am 27, April 1960

Es wurde aufgeklärt, daß die im Tee-blättern weit verbreitet vorkommende natürliche Blätter-alcohol-fraction aus einem Gemisch der *cis*, *trans*-Isomere besteht, wobei bisher das *cis*-Isomere besteht, wobei bisher das *cis*-Isomere stark überwiegt.

Das *cis*-, *trans*-Problem des natürlichen Blätteralkohols, das von M. Stoll[1] und S. Takei[2] seit 1938 bearbeitet wurde, konnte von L. Crombie[3] an Band des IR-Spektrums zugunsten der *cis*-Konfiguration entschieden wurden. Seither sind an der Konfiguration des natürlichen Blätteralkohols als 3-*cis*-Hexen-1-ol keine Zweifel mehr geäußert worden.

Wir konnten bei genauere Betrachtung des IR-Spektrums von L. Crombie eine sehr schwache Band bei 10.3 μ nicht übersehen. Zur genaueren Ermittlung dieser für eine *trans*-Doppelbindung charakteristischen Bande stellten wir natürlichen Blätteralkohol aus frischen Tee-blättern her. Diese Alkohol-fracktion (I) zeigte zwei scharfe Banden 10.3 μ (*trans*-Doppelbindung) sowie 11.5μ (*cis*-Doppelbindung) im IR-Spektrum (Abb. 1-a). Zur Untersuchungen der Frage, ob im Pflanzenreich von Anfang an bei *cis*- sowie *trans*-Zustand verbreitet vorkommen, order aber sich bei der Aufarbeitung aus der *cis*- zum Teil zu *trans*-Form umlagert, wurden folgende Versuche unternommen; 1) Synthetisches reine 3-*cis*-Hexen-1-ol wurde auf dem Ölbad 10 Stdn. auf 130~170° erhitzt. 2) Synthetisches reine 3-*cis*-Hexen-1-ol wurde in Benzol mit 3,5-Dinitrobenzoylchlorid und einigen Tropfen Pyridin versetzt und 3 Stdn. unter Rückfluß gekocht. Anschließend wurde das 3,5-Dinitrobenzoat durch Wasserdampfdestillation mit 40% KOH-lösung verseift. In beiden Aufarbeitungen zeigte das nach der Behandlung zurückgenommene 3-*cis*-Hexen-1-ol keine Änderung der Intensitäten im IR-Spektrum und in andern physikalischen Eigenschaften. Diese Ergebnisse zeigten, daß auch der natürliche Blätteralkohol unter den Bedingungen beständig sein sollte.

Als nächstes stellten wir aus natürlicher Blätteralkohol-fraktion (I) das 3,5-Dinitrobenzoat her. Durch vielfache systematische Fraktionierung gelang es uns, daraus den 3-*trans*-Hexen-1-ol-ester rein zu isolieren. Sein IR-Spektrum stimmte mit dem des synthetischen 3-*trans*-Hexen-1-ol-3,5-dinitrobenzoats völlig überein. Gegenüber dem synthetischen *cis*-Isomeren ergab sich es im IR-Spektrum deutliche Unterschiede (Banden bei 10.2~4 μ, 12.8~9 μ und 13.6~9 μ (Abb. 1-c, d)). Aus der systematischen Fraktionierung und dem IR-Spektrum scheint es hervorzugehen, daß das 3-*trans*-Hexen-1-ol zu 3~6% in der natürlichen (I) ist. Demnach besteht die natürlichen Blätteralkohol-fraktion (I) in Tee-blättern aus einem Gemisch der *cis*-, *trans*-Isomere, wodei das *cis*-Isomere stark überwiegt.

[†] VI. Mitteil: S. Takei und M. Ohno, *J. Agr. Chem. Soc. Japan*, **18**, 625 (1942),

[†] VII. Mitteil: A. Hatanaka, M. Hamada und M. Ohno, Dieses Bulletin, **24**, 115 (1960).

[††] Diese Mitteilung zu Notizen: *Naturforschung*, **15-b**, 415 (1960),

[1] M. Stoll und A. Rouve, *Helv. Chim. Acta*, **21**, 1542 (1938).

[2] S. Takei, M. Ohno und K. Schinosaki, *Ber.*, **73**, 950 (1940).

[3] L. Crombie und S.H. Harper, *J. Chem. Soc.*, **1950**, 873.

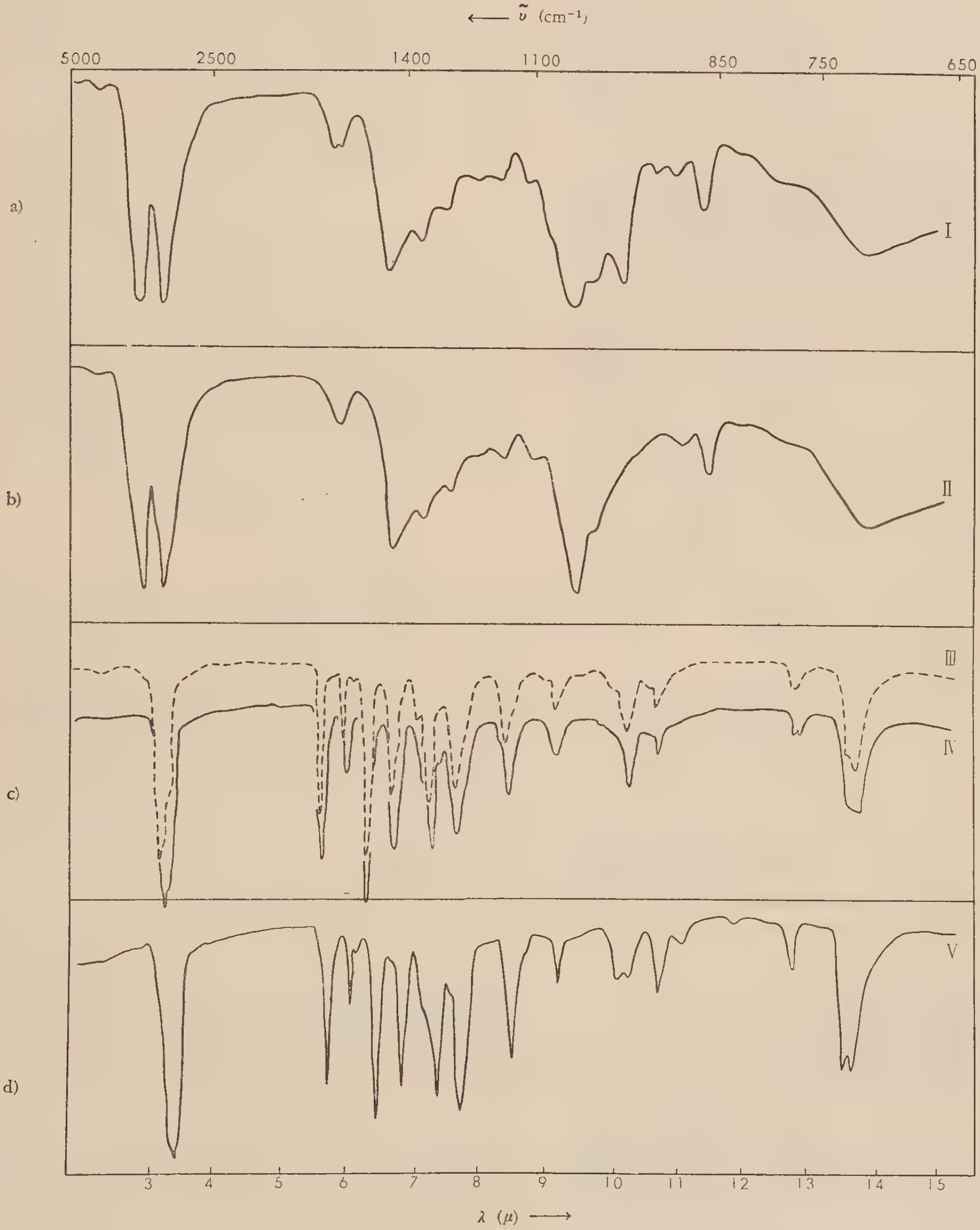

Abb. 1. IR-Spektren von a) Natürlicher Blätteralkokol-fraktion (I), b) Synthetische *cis*-Form (II), c) Natürliches *trans*-Ester (III) sowie Synthetisches *trans*-Ester (IV), und d) Synthetisches *cis*-Ester (V).

Mit dieser systematischen Fraktionierung gelang es uns, mit auch Gas-chromatograph aus der natürlichen Blätteralkohol-fraktion (I) *trans*-Isomeres rein zu isolieren, und daraus ging es hervor, daß *trans*-Isomeres zu 5% in der natürlichen (I) ist (Abb. 2).

BETRACHTUNGEN ÜBER INFRAROTSPEKTREN UND GAS-CHROMATOGRAMM

Aus dem IR-Spektrum vom natürlichen Blätteralkohol mit synthetischen 3-*cis*-Hexen-1-ol finden wir im zwischen $9\sim11\mu$ charakteristische Unterschieden; Die charakteristische Absorption bei $9.2\sim6\mu$ zeigt breite Band für die natürlichen Blätteralkohol-fraktion (I), gegenüber dem scharfe Band für synthetischen 3-*cis*-Hexen-1-ol (II) (Abb. 1-a, b). Aber wir können den gegauige Grund für diese Tatsache nicht erfinden und weiter wollen über das Problem untersuchen. Aus dem IR-Spektrum des synthetischen 3-*trans*-Hexen-1-ol-3,5-dinitrobenzoats (IV) und dieser *cis*-Form-esters (V) (Abb. 1-c,d) zeigt *trans*-Form eine Absorbtion und *cis*- zwei in zwischen $10.2\sim4\mu$, *trans*-Form zwei Absorbtion und *cis*- eine bei $12.8\sim9\mu$, und auch bei $13,6\sim9\mu$ die charakteristische Unterschieden.

Dieses IR-Spektrum des 3-*trans*-Hexen-1-ol-3,5-dinitrobenzoats (III), das aus dem natürlichen Blätteralkohol-fraktion-3,5-estern isoliert wurde, stimmte mit dem des synthetischen 3-*trans*-Hexen-1-ol-esters (IV) völlig überein (Abb. 1-c).

Zur Nachweis des *trans*-Isomeren in der natürlichen Blätteralkohol-fraktion (I) waren die Versuchsbedingungen: Trägergas: N_2 mit einer Strömungsgeschwindigkeit von etwa 45 ml/min; Temperatur: 130°; Kolonne (0.6 cm×3 m): Polyäthylen Glykol (6000); Spannung 2mV; Gerät: Schimazu GC-1A. (Abb. 2)

BESCHRIEBUNG DER VERSUCHE

Natürlicher Blätteralkohol (I):

3.3 Kg Frisches Tee-blätterchen, die sofort mit Pflücken zu kleinem Stück geschnitten wurden, wurden mit Wasser gleiches Volumens versetzt. Das Destillat, das durch die Wasserdampfdestillation gewannen wurde, wurde mit NaCl gesättigt, mit 3×500 ccm Äther ausgeschüttelt, und nachdem mit 2n-HCl sowie 2n-KOH gewaschen. Diese Aufarbeitung wurde in 30×je 3.3 Kg widerholt. Vom rohen ätherischen Öl aus ca. 95 Kg frischemTee-blätterchen siedeten 7.3 g Fraktion bei 68°/28 mm. Nach der Fraktionierung ging 4.29 g Fraktion bei 150~60° über. Diese Fraktion wurde mit 9.2 g Phthalsäureunhydrat in 20 ccm Benzol versetzt, und auf Wasserbad under Rückflußkühlung während 3 Stdn. gekocht. Nach der gewöhnlichen Behandlung wurden 11 g Phthalsäurehalbester erhalten. Nach der Wasserdampfdestillation mit 15% NaOH-lösung ging 3.08 g Fraktion (I) bei 152~54° aus dem Destillat über. n_D^{22} 1.4378. Isolierung 3-*trans*-Hexen-1-ol-3,5-dinitrobenzoats aus dem natürlichen Blätteralkohol-3,5-ester; Aus 2.02 g Fraktion (I) und 5.98 g 3,5-Dinitrobenzoylchlorid durch gewöhnliche Aufarbeitung wurden 6.0 g natürliches Blätteralkoholfraktion-3,5-ester hergestellt. Nach der vielfachen systematischen Fraktionierung aus Petroliumäther wurde 5 mg 3,5-Ester (7-A) (III) bei 43° isoliert und (III) stimmte mit (IV) im IR-Spektrum völlig überein. 3,5-Dinitrobenzoat (III); Schmp. 43°, $C_{13}H_{14}O_6N_2$ (294.27) Ber. C 53.06, H 4.76 Gef. C 53.29, H 4.96.

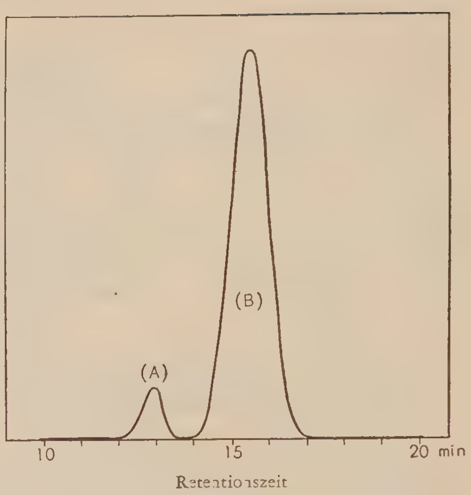

Abb. 2. Gas-chromatogramm der natürlichen Blätteralkohol-fraction (I)
(A): 3-*trans*-Hexen-1-ol
(B): 3-*cis*-Hexen-1-ol

3-Hexin-1-ol:

Nach Sondheimer[4] konnten aus 25 g Natrium, Acetylen, 155 g Äthyljodid und 90 g Äthylenoxyd

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[4] F. Sondheimer, *J. Chem. Soc.*, **1950**, 887.

in flüssigem Ammoniak 52 g 3-Hexin-1-ol (53% d.Th.) gewonnen wurden. Sdp. 89~92°/32 mm; 162~3°, n_D^{20} 1.4535. Reines 3-Hexin-1-ol wurde über dieses 3,5-Dinitrobenzoat oder Phthalsäurehalbester hergestellt. 3,5-Dinitrobenzoat; Schmp. 73°, $C_{13}H_{12}O_6N_2$ (292.25) Ber. C 53.46, H 4.14 Gef. C 53.26, H 4.17.

3-*trans*-Hexen-1-ol :

2 g Reines 3-Hexin-1-ol wurden in Natriumflüßigesammoniak-lösung (aus 1.9 g Natrium in 200 ccm flüßigem Ammoniak) unter Kühlung bei −40° und Rührung eingetropft. Nach 2 Stdn. entfärbt sich das Gemisch mit Zugabe von 6 g Ammoniumchlorid. 3-*trans*-Hexen-1-ol siedete bei 150~2°. n_D^{20} 1.4361. 3,5-Dinitrobenzoat (IV); Schmp. 47.5~8°, $C_{13}H_{14}O_6N_2$ (294.27) Ber. C 53.06, H 4.76 Gef. C 52.79, H 4.79.

3-*cis*-Hexen-1-ol :

2 g Reines 3-Hexin-1-ol wurden in 20 ccm Methylacetat mit 1.5 g Katalysator nach Lindlar[5] bis zur Aufnahme der ber. Wasserstoffmenge bei Raumtemperatur hydriert. Nach der Fraktionierung wurden 1.69 g (85% d.Th.) (II) erhalten. Sdp. 156°, n_D^{20} 1.4419. 3,5-Dinitrobenzoat (V); Schmp. 49.5~50°, $C_{13}H_{14}O_6N_2$ (294.27) Ber. C 53.06, H 4.79 Gef. C 52.78, H. 4.79.

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[5] H. Lindlar, *Helv. Chim. Acta*, **57**, 447 (1952).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 617~621, 1960]

Effect of Sodiumchloride in the Diet on the Occurrence of Liver Cancer

Induced by *p*-Dimethyl Amino Azobenzene (DMA)

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Department of Agricultural Chemistry, Faculty of Agriculture, Iwate University

Received May 16, 1960

The high concentration of sodiumchloride content in the diet has deleterious effects both on the occurrence of liver cancer induced by *p*-dimethyl amino azobenzene through decreasing riboflavin concentration of liver and on the production of hypertension in rats. The effects can be largely counteracted by the simultaneous feeding of riboflavin.

It is reported by Sugiura[1] that the riboflavin can prevent the outbreak of liver cancer induced by DMA in rats. Koyanagi et al[2]. have recognized that when riboflavin is added to the diet the amount of sodiumchloride elimination in the urine shows an increase. In another experiment they noticed that the increase of sodiumchloride in the diet from 1 to 2.5 g per 100 g of diet resulted in the decrease of riboflavin concentration in the liver[3]. Then the concentration of sodiumchloride in the diet may influence the occurrence of liver cancer in rats fed with diet having DMA. The purpose of

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[1] K. Sugiura, *J. Nutrition*, **44**, 345 (1951).

[2] T. Koyanagi et al., Annual Meeting of Japanese Vitamin Soc. (1958).

[3] T. Koyanagi et al., unpublished.

TABLE I. COMPOSITION OF DIETS

Group No.	Characteristics of diets	Milled Rice	Soybean	Sodium chloride	Calcium carbonate	DMA	Riboflavin
		g	g	g	g	mg	mg
1	Basal	83.5	15	0.5	1	—	—
2	DMA	83.5	15	0.5	1	60	—
3	DMA Sodiumchloride	82	15	2	1	60	—
4	DMA Sodiumchloride Riboflavin	82	15	2	1	60	2

TABLE II. INCREASE OF BODY WEIGHT, BLOOD PRESSURE AND RATIOS OF LIVER-, SPLEEN- AND ADRENAL-WEIGHT TO THE BODY WEIGHT

Group No.	Rat No.	Initial body weight	Final body weight	Increase of body weight	Blood pressure	Per 100g of body weight		
						Liver	Spleen	Adrenal
		g	g	g	Hg mm	g	g	mg
1	1	60	171	111	126	2.59	0.20	36
	2	64.5	174	109.5	126	2.44	0.20	26
	3	90	197	107	121	2.50	0.21	29
	Mean	71	182	109	124	2.51	0.21	30
2	4	77.5	154	76.5	130	4.41	0.76	44
	5	80.5	149	68.5	137			
	6	83.5						
	7	90	183	93	140	3.36	0.63	42
	8	71	180	109	137	3.37	0.66	30
	9	73	130	57	140	2.76	1.06	72
	Mean	79	159	80	137	3.56	0.77	46
3	10	84	139	55	130	4.35	0.66	40
	11	75	151	76	140	3.52	0.62	44
	12	90	133	43	147	3.63	0.60	42
	13	73	176	103	147	3.88	0.76	34
	14	80	146	66	147	3.55	0.61	44
	15	72.5	174	101.5	149	3.66	0.70	32
	Mean	79	153	74	143	3.76	0.66	40
4	16	68.5	182	113.5	122	3.18	0.29	34
	17	85.5	103	17.5	126	3.66	0.64	78
	18	90	88	−2	140	5.13	0.50	70
	19	70.5	184	113.5	143	2.73	0.33	32
	20	85.5	184	98.5	143	3.39	0.65	26
	21	70	165	95	143	3.94	0.53	34
	Mean	78.3	151	73.1	136	3.67	0.49	44

this paper is to present data indicating that sodiumchloride concentration in the diet is involved in the outbreak of liver cancer.

EXPERIMENTAL

Twenty-one male albino rats of the Wister strain, weighing 60～90 g, were divided into four groups and housed individually on wire screen and fed the following four diets:

group 1, a basal diet consisting of milled rice, defatted soybean meal, sodiumchloride and calciumcarbonate;

group 2, to the basal diet was added DMA;

group 3, DMA and sodiumchloride;

group 4, DMA, sodium chloride and riboflavin; also one drop of shark liver oil was supplemented weekly to each rat. The composition of the diets is shown in Table I.

DMA and riboflavin were mixed well with the diet, the former as a 3% solution in soybean oil, and the latter as 20 mg% solution in dilute acetic acid.

The diet was made into a paste by heating with 130 ml of water per 100 g and water was restricted to the amount necessary to make the paste. The amount of diet was 10 g per rat per day for the first two weeks and then increased by 2 g every three weeks.

After a nine weeks feeding period the blood pressure of the animals was determined by the Williams' rat tail method[4] and they were killed by decapitation. Riboflavin in liver, ascorbic acid in adrenals were determined by lumiflavin and indophenol method respectively, and liver tissue was examined microscopically.

RESULTS AND DISCUSSION

During the experiment the animals fed DMA grew less rapidly and one animal on the diet (2) died at the end of the eighth week.

As shown in Table II the blood pressure of rats fed DMA was higher than that of rats fed controlled diet. Among the former, rats kept on high salt diet had the highest blood pressure.

The increase of body weight of rats in the three groups fed with DMA was almost equal, but this increase was inferior to that of rats of controlled group. With the exception of those of the riboflavin supplemented group, liver, spleen and adrenals of rats on DMA diet hypertrophied severely.

From Table III it can be seen that the liver of rats of group (2) and (3) contained lower riboflavin concentration in comparison with the liver of rats of basal diet and among these groups rats on the high salt diet without riboflavin supplement have the lowest concentration. Ascorbic acid in the adrenals showed no significant difference between the groups.

As shown in the table, the occurrences of cancer in the liver were confirmed histologically in the rats of group (2) and (3).

Further, proliferations of cancer cells were generally more prominent in the livers of rats of the latter group fed with high salt diet than in the livers of the former group. On the contrary, no significant lesions were found in the livers of rats of group (4) together with those of the controlled group.

These pictures are presented at the end of this paper.

The high salt diet tends to decrease the riboflavin concentration of liver and consequently promotes the outbreak of cancer induced by DMA. Carcinogenecity is dependent on the concentration of riboflavin in liver[5]. Riboflavin added to the diet has strong protective effects against the pathological changes leading to the development of liver cancer.

TABLE III. CONCENTRATION OF RIBOFLAVIN AND ASCORBIC ACID IN LIVER AND ADRENALS RESPECTIVELY AND SCORE OF OCCURRENCE OF CANCER

Group No.	Rat No.	Riboflavin in liver	Ascorbic acid in adrenals	Scores of occurrence of cancer in liver
		mg%	mg%	
1	1	1.78	253	−
	2	2.43	242	−
	3	2.11	245	
	Mean	2.11	246	
2	4	3.10	249	+
	5	1.63	255	+
	6	—		
	7	2.33	198	+
	8	1.51	243	+
	9	1.25	232	+
	Mean	1.96	235	
3	10	1.60	217	−
	11	1.03	224	+
	12	1.49	236	+++
	13	1.21	201	+++
	14	0.81	266	+++
	15	1.13	327	+++
	Mean	1.21	245	
4	16	2.35	249	−
	17	—	253	−
	18	1.89	308	−
	19	2.10	250	−
	20	1.82	297	−
	21	1.78	244	−
	Mean	1.99	267	

Note: Scores of occurrence of cancer in liver were based chiefly on the degrees of proliferation of cancer cells.

− : No significant changes are noted.

+ : Slight proliferation of cancer cells noted chiefly in interlobular areas.

+++: Marked proliferation of cancer cells noted not only in Glisson's capsules but also in some of sinusoids. Interlobular areas are filled densely with these cell masses, causing pressure atrophy of hepatic parenchyma.

Blood pressure of rats on DMA diet was higher than that of rats on the controlled diet. Among the former, rats fed with high salt diet showed the highest blood pressure.

Thus high concentration of sodiumchloride in the diet seems to stimulate both the appearance of high blood pressure and the outbreak of liver cancer.

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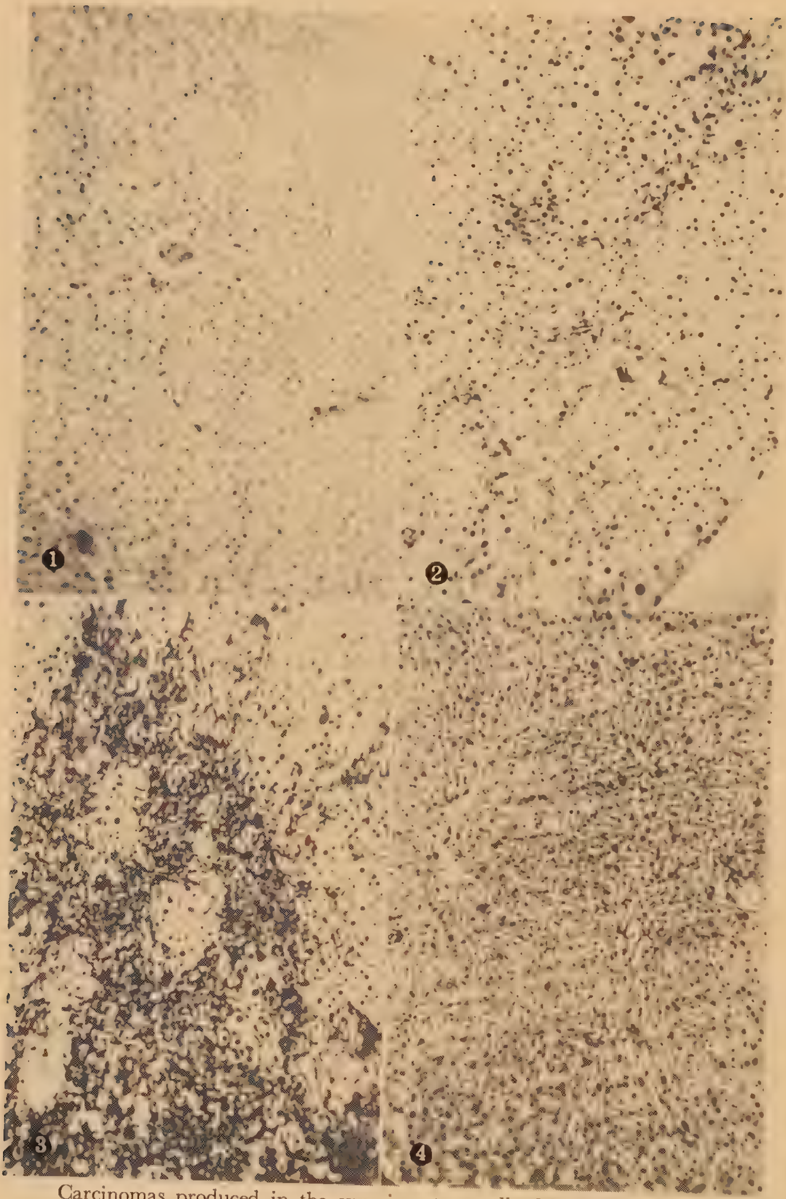
[4] T. Koyanagi et al., This Bulletin, **23**, 3 (1959).

[5] E.C. Miller, J.A. Miller, B.E. Klinie and H.P. Rusch, *J. Exp. Med.*, **88**, 89 (1948).



Carcinomas produced in the experiment are all of bile-duct type.

FIG. 1. (Control). Figure of normal liver in group (1). H-E stain, ×110.

FIG. 2. (DMA). Liver of rat (No. 4) in group (2), showing slight proliferation of cancer cells chiefly in interlobular areas. The cell proliferation occurs especially in the peripheral portion of the liver. H-E stain, ×110.

FIG. 3. (DMA, Sodiumchloride). Liver of rat (No. 12) in group (3). Marked proliferation of cancer cells is noted in the interlobular areas and in some of sinusoids, especially in the peripheral portion of the liver. Islet-like hepatic tissues are found among these proliferated cell masses. The formation of typical bile-duct carcinoma is also noted in other portion. H-E stain, ×110.

FIG. 4. (DMA, Sodiumchloride, Riboflavin). Liver of rat (No. 17) in group (4). No significant changes are noted. H-E stain, ×110.

It is a well-established fact that the incidence of primary cancer of the liver among Orientals who consume rice as staple food is very much higher than the incidence of this cancer among Europeans and Americans. And one cause has been attributed to the lower riboflavin content of rice diet than that in the bread and milk diet¹⁾. As the rice diet requires much more sodiumchloride for seasoning than the bread milk diet, another cause may be the high salt consumption of the former.

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Studies on the Amino Acids Fermentation of Pentose Materials

Part I. Isolation of strains capable of producing amino acids from pentose and their taxonomical studies

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Isolation and screening tests were carried out to obtain microorganisms capable of producing amino acids from pentoses and hexoses directly in the presence of nitrogen sources and inorganic salts.

Three strains having exceedingly high amino acids producing ability were obtained. One of them produces remarkably L-glutamic acid and alanine and the other two have high productivity of alanine.

As a result of the taxonomical studies, all of these strains were found to belong to the Genus *Brevibacterium*. Moreover, there are no species which conform to these strains described in Bergey's Manual of Determinative Bacteriology 7th Ed., so the authors decided them to be new species.

INTRODUCTION

It has been generally recognized that fermentation process for the production of amino acids has many advantages compared with the isolation methods of hydrolyzed proteins or synthetic methods. And at present some amino acids such as L-glutamic acid are actually produced by fermentation process on a commercial scale. Moreover, there are many amino acids which have high possibilities to be manufactured industrially by fermantation in the near future.

But the fermentation of these amino acids seem to have the limitation with regard to the kinds of carbon sources utilized. With the exceptions of α-ketoglutaric acid¹⁾, fumaric

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1) S. Otsuka, H. Yazaki, H. Nagase and K. Sakaguchi, This Bulletin, **21**, 69 (1957).

acid[2], acetic acid[3], γ-aminobutyric acid[4] and 3-indolepyruvic acid[5] (these acids resemble to the products in their chemical structures or seem to be related to the intermediates in the pathways of their biosyntheses from sugars), glucose[6,7] is the only carbon source utilized especially on a commercial scale.

It is true that glucose is the most abundant and commonest sugar among the natural resources, however, it can not be neglected that there are many other sugars such as pentoses. To use the natural resources more effectively, it seems to be important to take these sugars into account other than glucose.

If certain microorganism is able to utilize not only hexoses but pentoses producing some amino acids directly, the fermentative production by this microorganism has a great advantage in the utilization of the wastes of agriculture and forestry such as wood hydrolysates and sulfite waste liquor.

On this intention, we have attempted to screen the microorganisms which would produce amino acids from pentose. As a result of these tests we found three new species of *Brevibacterium* which have exceedingly high potencies: one of them has an ability of producing L-glutamic acid and alanine and others are capable of producing alanine.

EXPERIMENTAL AND RESULTS

(I) Isolation of strains capable of producing amino acids from pentoses.

Microorganisms were isolated from a variety of soil samples in such places as fields, forests, greens and sewages. About 0.1 g of the sample was supended in 5 ml of sterilized water and the suspension was plated out on the agar media composed of the materials mentioned in Table I.

TABLE I. CULTURE MEDIUM EMPLOYED FOR ISOLATION

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Xylose, 2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K_2HPO_4, 0.1%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$MgSO_4$7aq., 0.05%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urea, 0.3%	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
KNO_3, 1.0%	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
NH_4Cl, 0.6%	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−
Casate, 0.02%	−	−	+	+	−	−	+	+	−	−	−	−	+	+	+	+	+	+
Peptone, 0.2%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Meat extract, 0.2%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Yeast extract, 0.2%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Agar, 3.0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH	7	8	7	8	7	8	7	8	7	7	8	8	7	7	8	8	7	8
$CaCO_3$, 1.0%	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+	−	−

In the earlier stage of these experiments, the pH was adjusted to 7.0, but under this condition the growth of fungi is too heavy to pick up the colonies of bacteria, so the pH was adjusted to 8.0 afterwards.

After 72 hours incubation at 30°C, the organism grown on the agar plates was transferred to the slant culture having the same medium composition as the plate culture. It was inoculated into the 5 ml of liquid medium in the test tube, the composition of which is shown in Table II, and cultivated aerobically by the reciprocal shaker for 72 hours at 30°C.

Having finished the incubation, the culture broth

TABLE II. CULTURE MEDIUM EMPLOYED FOR SCREENING TESTS

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Xylose, 3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K_2HPO_4, 0.1%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$MgSO_4$ 7aq., 0.05%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urea, 0.4%	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
KNO_3, 1.3%	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−
NH_4Cl, 0.7%	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Casate, 0.02%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Peptone, 0.2%	−	+	−	−	−	+	−	+	−	−	−	+	−	+	−	−	−	+
Meat extract, 0.2%	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+
Yeast extract, 0.2%	−	−	−	+	−	+	−	−	−	+	−	+	−	−	−	+	−	+
C.S.L., 0.2%	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+
$CaCO_3$, 1.0%	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
pH	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7

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[2] R. Aoki, Y. Kondo, T. Tsunoda and T. Ogawa, *J. Agr. Chem. Soc. Japan*, **33**, 843 (1959).

[3] I. Shio, S. Otsuka, T. Tsunoda, Symposium on the Enzyme Chemistry (1959).

[4] T. Tsunoda, R. Aoki, K. Kinoshita and Y. Kondo, *J. Agr. Chem. Soc. Japan*, **33**, 221 (1959).

[5] K. Aida, T. Asai, T. Kajiwara and K. Iizuka, *J. Gen. Appl. Microb.*, **4**, 200 (1958).

[6] T. Asai, K. Aida and K. Oishi, This Bulletin, **21**, 134 (1957).

[7] S. Kinoshita, S. Udaka and M. Shimono, *J. Gen. Appl. Microb.*, **3**, 193 (1957).

was centrifuged to remove the cells and the determination of amino acids was made by paper chromatography. The solvent used was a mixture of *n*-butanol, acetic acid and water (4:1:4 by vol.), and 0.2% ninhydrin *n*-butanol solution was employed to color the spot on paper chromatogram. The standard amino acids solutions were simultaneously developed on the paper to identify the spots and to estimate the concentration of amino acids roughly. The microbiological methods and the manometric method using a glutamic decarboxylase were also adopted for the quantitative analysis.

The results of the screening tests were summarized in Table III.

TABLE III. RESULTS OF THE SCREENING TESTS FOR AMINO ACIDS PRODUCTION FROM XYLOSE BY BACTERIA

Amino acid production	Numbers of the strains	Ratio
++	4	0.4%
+	62	5.5
−	1060	94.3
Total	1126	100

++ : >3mg/ml
+ : 3 mg/ml> >1 mg/ml

Out of the 1126 strains isolated only four strains could produce amino acids with a concentration higher than 3 mg per ml of culture broth. The amino acid produced by these four strains was exclusively alanine.

With the newly isolated strains mentioned above, we examined about 150 strains of type cultures preserved in the Institute of Applied Microbiology, University of Tokyo. The genera examined were *Pseudomonas*, *Aerobacter*, *Xanthomonas*, *Vibrio*, *Agrobacterium*, *Alcaligenes*, *Achromobacter*, *Flavobacterium*, *Escherichia*, *Aerobacter*, *Erwinia*, *Serratia*, *Proteus*, *Micrococcus*, *Sarcina*, *Brevibactirium*, *Corynebacterium*, *Microbacterium*, *Bacillus*, *Mycobacterium* etc. In type cultures, only *Brevibacterium helvolum* could produce alanine from pentose with a yield of few per cent for sugar, and very small amounts of L-glutamic acid, alanine and aspartic acid were formed by some strains of *Micrococcus*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Escherichia*, *Aerobacter* and *Bacillus*.

At the same time we examined several strains of stock cultures of our laboratory and of Dr. Arima's, University of Tokyo, and we found one of the strains of Dr. Arima has an strong ability of producing L-glutamic acid and alanine from pentose.

Thus the screening tests were successful in finding out five strains of exceedingly high amino acids productivity and three of them, strains No. 115-1, No. 2398 and No. 2405, which have especially high ability of producing amino acids, have been identified to the Genus *Brevibacterium*. Taxonomical studies of these strains will be shown in the following paragraphs.

(II) Taxonomical studies of strains No. 115-1, No. 2398 and No. 2405. The strains No. 115-1, No. 2398 and No. 2405 were tested diagnostically. These tests were performed according to the "Manual of Microbiological Methods', 1957, Society of American Bacteriologists, and referred to "An Introduction to Laboratory Technique in Bacteriology", by the same authors as above.

Temperature for incubation was generally maintained at 30°C in most cases, except in the case of gelatin liquefaction test.

(1) The properties of strains No. 115-1, No. 2398 and No. 2405.

The characters of these strains are as follows;

I) Microscopic observation: In the case of strain No. 115-1, cells are straight rods with rounded ends, 0.5 to 1.0, by 1.0 to 2.0 micron. Usually single, occasionally in pairs or irregular masses. No capsules or endospores observed. Motile by flagella and gram-positive. Not acid-fast. Granulations not demonstrable with methylene blue stain (Fig. 1).

As for strains No. 2398 and No. 2405, cells are straight rods with rounded ends, 0.4 to 0.6, by 1.0 to 1.5 micron. Usually in pairs or in chains, occasionally irregular masses. No capsules or endospores observed. Gram-positive. Non-motile. Not acid-fast and granulations not demonstrable with methylene blue strain.

II) Agar colonies: The observations on agar colonies of these strains are as follows:

Moderate growth, punctiform, smooth, raised, glistning, yellow (the yellow color is much deeper in the case of strain No. 115-1, and strains No. 2398 and No. 2405 show yellowish white color), non-viscid. Edge is entire.

III) Agar slant: All of these strains show the following characters: Moderate growth, yellow filiform, glistning, non-viscid. Medium unchanged. The mycelium of strain No. 115-1 is found to show slight fluorescence.

IV) Gelatin stab: All of these strains liquefy gelatin considerably.

V) Nutrient broth: On this test too, there is no remarkable difference among these strains. Slightly turbid, yellowish flocculent sediment, no-odor.

TABLE IV. THE PHYSIOLOGICAL PROPERTIES OF STRAINS No. 115-1, No. 2398, No. 2405.

Temperature relations:	Heavy growth between 28° and 32°C. Thermal death point at 70°C for 15 minutes.		
Litmus milk:	No change	No change	Alkali
Indole formation:	−	−	−
Nitrate reduction:	−	+	+
Ammonia formation in peptone water:	+	+	+
H_2S formation:	+	+	−
Methyl red test:	−	−	−
Voges-Proscauer test:	−	−	−
Catalase:	+	+	+
Urease:	+	+	+
Reaction to free O_2:	aerobic	aerobic	aerobic
pH relation:	Heavy growth between 7.0 and 8.0.		

TABLE V. ACID AND GAS FORMATION FROM CARBOHYDRATES BY STRAINS No. 115-1, No. 2398, No. 2405.

Carbohydrates	No. 115-1	No. 2398	No. 2405
Arabinose	+	+	+
Rhamnose	−	−	+
Xylose	+	+	+
Glucose	+	+	+
Fructose	+	+	−
Galactose	±	−	−
Mannose	±	+	+
Lactose	−	−	−
Sucrose	−	−	+
Maltose	−	−	−
Starch	−	−	−
Inulin	−	−	−
Dextrin	−	−	−
Glycerol	−	−	±
Mannit	−	−	−
Salicin	−	−	−

+ : Acid formed. − : Acid not formed.
No gas is produced in all cases.

VI) Physiological properties: Table IV and V show the physiological proporties of these strains.

Strain No. 115-1 has a strong ability of accumulating L-glutamic acid and alanine in a large quantity aerobically in the presence of pentoses or hexoses, ammonium ion and inorganic salts. And strains No. 2398 and No. 2405 are able to produce exceedingly high amounts of alanine from pentoses and hexoses with ammonium ion and inorganic salts in the aerobic condition.

(2) Decision of family.

These strains are gram-positive, unbranched straight rods and incapable to form endospores. Therefore, they belong to Family *Brevibacteriaceae* described in "Bergey's Manual of Determinative Bacteriology" 7th Ed., 1957.

They are not spherical, so there is no possibility that they belong to Family *Micrococcaceae* or Family *Neisseriaceae*. If our strains are regarded as wedge or club forms, then they may belong to Family *Corynebacteriaceae*. But they do not show these forms and all species in this family are not in accord with them. Moreover, having aerobic properties, they cannot belong to Family *Lactobacillaceae* or Family *Propionibacteriaceae*.

(3) Decision of genus.

In Family *Brevibacteriaceae* there are two genera, i.e., *Brevibacterium* and *Kurthia*. According to the descriptions in Bergey's Manual, *Kurthia* is long, unbranched rod which may form filaments and does not utilize carbohydrates. Our strains are short rods and do not form filaments and utilize carbohydrates. Consequently they cannot belong to *Kurthia*.

In relation to *Brevibacterium*, following descriptions can be seen in Bergey's Manual: "Typically short, unbranching rods. May or may not reduce nitrates. Glucose broth usually becomes acid; lactose not fermented. Aerobic and facultatively anaerobic". Our strains conform to all of these descriptions. Thus, they were confirmed to the Genus *Brevibacterium*.

(4) Decision of species.

There are seventeen non-motile and six motile species in Genus *Brevibacterium* and the comparisons between our strains and these named *Brevibacterium* species are shown in Table VI and VII.

One of our strains, No. 115-1, is motile by flagella, so each species other than *Brev. incertum*, *Brev. imperiale*, *Brev. lipolyticum*, *Brev. acetylicum*, *Brev. sulfureum* and *Brev. helvolum* are different from strain No. 115-1 in their non-motile character. *Brev. incertum* and *Brev. imperiale* are different from strain No. 115-1 in the respect that they do not liquefy gelatin. *Brev. lipolyticum* is also differentiated from strain No. 115-1 in its grayish-white color on agar slant and also in several physiological characters. *Brev. acetylicum*, *Brev. sulfureum* and *Brev. helvolum* are different from strain No. 115-1 in their behavior to milk.

In contrast with strain No. 115-1, strains No. 2398 and No. 2405 are non-motile, so these strains cannot be identified as all the motile species mentione above. Among the non-motile species, *Brev. linens*, *Brev. erythrogenes*, *Brev. fulvum*, *Brev. insectiphilium*, *Brev. brunneum*, *Brev. vitarumen*, *Brev. maris* and *Brev. fuscum*

TABLE VI. COMPARATIVE STUDIES ON PHYSIOLOGICAL CHARACTERISTICS OF OUR STRAINS AND OTHER NAMED *Brevibacterium* SPECIES

Tests	1	2	3	4	5	6	7	8
Brev. linens.	−	+	−	Al	+	−	+	
Brev. erythrogenes.	−	+	+	Al	+	±	+	
Brev. fulvum.	−	−	+	A	+	+		
Brev. insectiphilium.	−	−		Al	+	−	−	
Brev. brunneum.	−	−		N	+	−		
Brev. vitarumen.	−	+		A	−	−		
Brev. maris.	−	+		Al-A	−	−	−	
Brev. fuscum.	−	+		A-Al	+	−		
Brev. minutiferula.	−	−		N-A	−	−	−	+
Brev. sociovivum.	−	−	+	N	+	−	−	+
Brev. immotum.	−	−	+	N	+	−	−	+
Brev. marinopiscosum.	−	−	+	N	+	−	−	+
Brev. tegumenticola.	−	−	−	N	−	−	−	−
Brev. stationis.	−	+		Al	+	−	−	+
Brev. quale.	−	+		N	+	−	−	−
Brev. ammoniagenes.	−	+		Al	−	−		−
Brev. healii.	−			A	+	−		
Brev. incertum.	+	−	−	N	−	−	−	
Brev. imperiale.	+	−		N-A	−	−	−	
Brev. lipolyticum.	+		+	Al	+	±	−	
Brev. acetylicum.	+	−	+	A	+	−		
Brev. sulfureum.	+	−	+	Al	+	−		
Brev. helvolum.	+	−	+	A-Al	+	−	++	
Brev. No. 115-1	+	−	−	N	+	−	+	
Brev. No. 2398	−	+	−	N	+	−	+	
Brev. No. 2405	−	+	−	Al	+	−	−	

Test 1: Motility, 2: Nitrate reduction, 3: Coagulation of milk, 4: Reaction of milk, 5: Gelatin liquefaction, 6: Indole formation, 7: Hydrogen sulfide formation, 8: Sea water requirement for initial growth.
N: No change, A Acid, Al: Alkaline.

TABLE VII. COMPARATIVE STUDIES ON CARBOHYDRATES UTILIZATION OF OUR STRAINS AND OTHER NAMED *Brevibacterium* SPECIES

Carbohydrates	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Brev. linens.	−	−	−	−	−	−		−	−	−		−	−		−	−
Brev. insectiphilium.	−	−	−	−	−	−	−	−	−	−		−	−	−	−	−
Brev. brunneum.				−				−	−							
Brev. vitarumen.			−	+				−	+	+					−	
Brev. maris.				+				−	−							
Brev. minutiferula.				+				−	+	−						
Brev. sociovivum.				+				−	−	+				−	+	−
Brev. immotum.			+	+				−	−	+				−	+	−
Brev. marinopiscosum.			−	+				−	−	−				−	+	−
Brev. tegumenticola.				+				−	+	+						
Brev. stationis.			−	+				−	−	−				−	−	−
Brev. quale.				+				−	+	+						
Brev. healii.				+	+			−	+	+	+	−			−	+
Brev. incertum.		−		+	+	−	+	−	+	+					−	
Brev. imperiale.	+	−	+	+	+	+	+	±	+	+		−	±	−	+	+
Brev. lipolyticum.			+	+				−	+					+	+	
Brev. acetylicum.				+												
Brev. sulfureum.				−												
Brev. helvolum.	+		+	+	+				+					+	+	
Brev. No. 115-1.	+	−	+	+	+	±	±	−	−	−	−	−	−	−	−	−
Brev. No. 2398.	+	−	+	+	+	−	+	−	−	−	−	−	−	−	−	−
Brev. No. 2405.	+	+	+	+	−	−	+	−	+	−	−	−	−	±	−	−

A: arabinose, B: rhamnose, C: xylose, D: glucose, E: fructose, F: galactose, G: mannose, H: lactose, I: sucrose, J: maltose, K: starch, L: inulin, M: dextrin, N: glycerol, O: mannit, P: salicin.

differ from these in their red, rose, green, brown or orange color on agar slant and in several physiological characters. *Brev. minutiferula*, *Brev. sociovivum*, *Brev. immotum*, *Brev. marinopiscosum* and *Brev. tegumenticola* are different from these in the respect that they cannot reduce nitrates to nitrites. *Brev. stationis* is also different in its sea water requirement for initial growth. *Brev. quale* is differentiated in the behavior to milk, the formation of hydrogen sulfide and so on. *Brev. ammoniagenes* is different in the gelatin liquefaction test. *Brev. healii* is different in the action to milk.

Therefore, there are no species which conform to either strain No. 115-1 or No. 2398 or No. 2405 in Beagey's Manual 7th Ed., so we decided them to be of new species. And we propose to assign the name, *Brevibacterium pentoso-aminoacidicum* to strain No. 115-1 in relation to its remarkable character of being able to produce amino acids from pentose, and the name, *Brevibacterium pentoso-alanicum* to strains No. 2398 and No. 2405 because of their ability of producing alanine from pentose.

(5) Comparisons between these new species of *Brevibacterium* and several amino acids producing bacteria.

Being stated already, many species of bacteria are known to be able to produce L-glutamic acid or alanine from glucose, but unfortunately, the morphological properties of only six strains of them have been reported. Therefore we could compare our strains with these species of bacteaia. The summary of the comparisons on their properties are shown in Table VIII and IX.

It is evident that our strains are quite different from all of these L-glutamic acid or alanine producing bacteria in respect to the forms of cells and several

TABLE VIII. PHYSIOLOGICAL PROPERTIES OF OUR STRAINS AND OTHER L-GLUTAMIC ACID OR ALANINE PRODUCING BACTERIA

Tests	1	2	3	4	5	6
Micrococcus glutamicus.[8)]	−	+		Al	−	−
Micrococcus varians.[9)]	−	+	+	Al	−	
Brevibacterium divaricatum.[10)]	−	−	+	A	−	−
Brevibacterium alanicum[11)]	−	+		A	−	−
Brevibacterium aminogenes.[12)]	−	+		Al	−	−
Microbacterium sp.[13)]	−	+	−	N	−	−
Brev. pentoso-aminoacidicum.	+	−	−	N	+	+
Brev. pentoso-alanicum. No. 2398	−	+	−	N	+	+
Brev. pentoso-alanicum. No. 2405	−	+	−	Al	+	−

Test 1: Motility, 2: Nitrate reduction, 3: Coagulation of milk, 4: Reaction of milk, 5: Gelatin liquefaction, 6: Hydrogen sulfide formation.
N: No change, A: Acid, Al: Alkaline.

physiological characters.

But the more important difference is the kinds of carbon sources utilized: *Brev. pentoso-aminoacidicum* No. 115-1 has an ability of prodncing remarkable amounts of L-glutamic acid and alanine from xylose, arabinose, glucose and fructose. *Brev. pentoso-alanicum* No. 2398 can produce alanine from xylose, arabinose, glucose, mannose and galactose remarkably. *Brev. pentoso-alanicum* No. 2405 can utilize xylose, arabinose, glucose, fructose, mannose and galactose with high alanine productivity.

Descriptions of new species.

1. ***Brevibacterium pentoso-aminoacidicum*:**

Rods, 0.5 to 1.0 by 1.0 to 2.0 micron, with rounded ends, occuring singly, in pairs and in irregular masses. Motile by flagella. Gram-positive. No capsules pa endospores observed.

Agar colonies: Moderate growth, punctiform, smooth, raised, glistning, yellow, non-viscid. Edge is entire.

Agar slant: Moderate growth, yellow, filiform, glistning, non-viscid. Medium unchanged.

Gelatin stab: Liquefaction.

Nutrient broth: Slightly turbid, yellowish flocculent sediment, no odor.

Litmus milk: No change.

Indole not produced.

Nitrites not produced from nitrates.

TABLE IX. CARBOHYDRATES UTILIZATION OF OUR STRAINS AND OTHER L-GLUTAMIC ACID OR ALANINE PRODUCING BACTERIA

Carbohydretes	A	B	C	D	E	F	G	H	I	J
Micrococcus glutamicus.	±	−	±	+	−	+	−	+	+	−
Micrococcus varians.	−	+	+	−	+	+	+	−		−
Brevibacterium divericatum.	−	−	−	+	−	+	−	+	+	+
Brevibacterium alanicum.	−		−	−	−	−	−	±	+	−
Brevibacterium aminogenes.	±			±	±			+	±	−
Microbacterium sp.	±		±	+	±	+	−	+	−	±
Brev. pentoso-aminoacidicum.	+	−	+	+	±	±	−	−	−	−
Brev. pentoso-alanicum. No. 2398	+	−	+	+	−	+	−	−	−	−
Brev. pentoso-alanicum. No. 2405	+	+	+	−	−	+	−	+	−	±

A: arabinose, B: rhamnose, C: xylose, D: fructose, E: galactose, F: mannose, G: lactose, H: sucrose, I: maltose, J: glycerol.

Ammonia produced from peptone.

Hydrogen sulfide is produced.

Methyl red test negative.

Voges-Proskauer test negative.

Catalase positive.

Urease positive.

Aerobic, facultatively anaerobic.

Acid but no gas from arabinose, xylose, glucose and fructose. No acid from rhamnose, lactose, sucrose, maltose, starch, inulin, dextrin, glycerol, mannit and salicin.

Remarkable amounts of L-glutamic acid and alanine are produced from xylose, arabinose, glucose and fructose in the presence of ammonium ion and other inorganic salts aerobically.

Optimum temperature, between 25° and 35°C.

Source: Found in sewages.

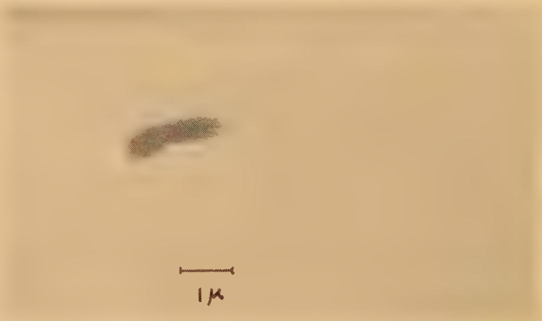


FIG. 1. *Brevibacterium pentoso-aminoacidicum*

2. ***Brevibacterium pentoso-alanicum*:**

Rods, 0.4 to 0.6 by 1.0 to 1.5 micron, with rounded ends, occuring in pairs, in chains and in irregular masses. Non-motile. Gram-positive. No

---

8) S. Kinoshita, K. Nakayama and S. Akita, This Bulletin, **22**, 176 (1958).

9) T. Asai, K. Aida and K. Oishi Bulletin, **21**, 134 (1957).

10) Y. Su and K. Yamada, This Bulletin, **24**, 69 (1960).

11) T. Ogawa, T. Oide and Y. Midorikawa, *Amino Acids*, **1**, 45 (1959).

12) S. Ota and M. Tanaka, *Amino Acids*, **1**, 50 (1959).

13) S. Doi, Symposium on the Amino Acids Fermentation (1959).

capsules or endospores observed.

Agar colonies: Moderate growth, punctiform, smooth, raised, glistning, yellow, non-viscid. Edge is entire.

Agar slant: Moderate growth, yellow, filiform glistning, non-viscid. Medium unchanged.

Gelatin stab: Liquefaction.

Nutrient broth: Slightly turbid, yellowish flocculent sediment, no odor.

Litmus milk: No change or alkaline.

Indole is not produced.

Nitrites produced from nitrates.

Ammonia preduced from peptone.

Hydrogen sulfide is produced or not produced.

Methyl red test negative.

Voges-Proskauer test negative.

Catalase positive.

Urease positive.

Aerobic, facultatively anaerobic.

Acid but no gas from arabinose, xylose and glucose. No acid from galactose, lactose, maltose, starch, inulin, dextrin, mannitol and salicin.

Remarkable amounts of alanine are produced from xylose, arabinose, glucose, mannose and galactose in the presence of ammonium ion and other inorganic salts aerobically.

Optimum temperature, between 25° and 35°C.

Source: Found in soils.

Acknowledgements. We are very grateful to Prof. K. Arima and Dr. K. Yano of Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo and Mr. K. Komagata of the Institute of Applied Microbiology, University of Tokyo for their kindness in making available their stock cultures.

Short Communications

Metabolic Products of *Oospora* sp.

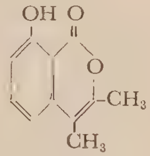
Sir:

The present communication is described on three compounds obtained from the mycellium and the culture filtrate of *Oospora* sp.. The studies on the metabolic products of *Oospora* are an underdeveloped field and few metabolits have been reported previously[1,2]. The auther's microorganism was isolated from the air with Waksman's and Sabouraud's agar.

The microorganism was cultivated on a medium containing the following ingredients as gram per liter; glucose 20, malt extract 20, and peptone 1. The incubation temperature and period were 25°C and 30 days respectivily. The dried mycellium was extracted with acetone and this extract was further devided into two parts by treating with hot petroleum benzene.

Crude 0-1 was obtained from soluble portion and recrystallized several times from ethanol as colorless needles, m.p. 129°C. *Anal.* Found: C, 69.59; H, 5.26%, M.W.: 184 (Rast). Calcd. for $C_{11}H_{10}O_3$: C, 69.60; H, 5.37%, M.W. 190.1.

0-1 was a new compound and the authors assumed the following structure by the chemical experiments.



This structure resembles to mellein (ochracin), which was isolated from the culture filtrated of *Aspergillus melleus*[3] and *Asp. ochraceus*[4].

The 0-3 compound was obtained from the insoluble part by hot petroleum benzene. It was recrystallized with methanol as colorless needles, m.p. 280°C and $[\alpha]_D^{17}=+34°$ (pyridine). *Anal.* Found: C, 78.90; H, 10.77%, M.W. 465.8 (Rast). Calcd. for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71%, M.W. 470. This 0-3 compound is now supposing as eburicoic acid[5] by the several experiments.

The filtrate was treated with carbon and this absorbed carbon cake was extracted with methyl acetate. From this extract, the 0-2 compound was isolated, and recrystallized several times from ethanol as the colorless needless, m.p. 176°C. *Anal.* Found: C, 59.90; H, 3.66%, M.W. 240 (Rast). Calcd. for $C_{11}H_8O_5$: C, 60.00,; H, 3.66%, M.W. 220.

This new compound has antibiotic properties to *Staph. aureus* 209-p, *E. coli* 0-112 and *Shigera flexine* EW.-9 as per one mililiter one miligram.

The classification of this microorganism and the determination of 0-2 structure are now proceeding.

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Kobe Municipal Hygienic Labolatory,
Kobe, Japan.

Received August 1, 1960

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1) H. Nisikawa, This Bulletin, **12**, 1 (1936); **13**, 1 (1937); *Acta Phytochem.*, **11**, 167 (1939).

2) F. Kögl, G.C. von Wessem, *Rec. Trav. Chim.*, **63**, 5 (1944).

3) H. Nisikawa, *J. Agr. Chem. Soc.* (*Japan*), **9**, 772, 1059 (1933).

4) T. Yabuta and Y. Sumiki, *J. Agr. Chem. Soc.* (*Japan*), **9**, 1264 (1933); **10**, 703 (1934).

5) T. Kariyone and G. Kurono, *J. Pharm. Soc.* (*Japan*), **60**, 318 (1940).

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 6, p. 629~630, 1960]

Muta-aspergillic Acid, A New Growth Inhibitant against Hiochi-bacteria

Sir;

In the previous paper[1], the author isolated hydroxyaspergillic acid, $C_{12}H_{20}N_2O_3$, m.p. 152~3°C, from the culture filtrate of *Aspergillus oryzae* as a growth inhibitant against hiochi-bacteria.

From the pre-analysis material of this acid, the author, recently, has succeeded to isolate the other new growth inhibitant against hiochi-bacteria by counter current distribution between ethyl acetate and Sörensen's N/10 citrate buffer solution (pH 6.2) in a yield of about one percent.

The author proposes to designate this antibiotic as muta-aspergillic acid.

Muta-aspergillic acid was recrystallised as a pale yellow needle from aqueous alcohol. It is readily soluble in most organic solvent, and slightly soluble in ether, *n*-hexane and hot water, but insoluble in petroleum ether and ligroin. It melts at 173~4°C (uncorr.)(decomposed and sublimes), and is optically inactive. $[\alpha]^{25}_{D}=0\pm0.3°$ c=3.5 in methanol). *Anal.* Found; C 57.67, H 8.44, N 12.25, O 21.64%, M.W. 246 (determined by Rast method) Calcd. for $C_{11}H_{18}N_2O_3$; C 58.39, H 8.02, N 12.38, O 21.21%, M.W. 236. Kuhn-Roth C-methyl determination gave 1.9 moles of acetic acid per mole. Ultra-violet absorption in methyl alcohol shows the 2 bands, λ_{max} 242 mμ (ε=5140) and λ_{max} 335 mμ (ε=8571) and pKa value of 4.8 was measured. Ninhydrin and ferric chloride reactions were both positive. It formed emerald green cupric salt melting at 231°C.

These properties and infrared spectrum shown in Fig. 1 suggest that this antibiotic belongs to the group of aspergillic, hydroxyaspergillic[2~4], and neohydroxyaspergillic[5] acids, which have the same basic structure of cyclic hydroxamic acid. However, the molecular formula of muta-aspergillic acid was found to be less one methylene group than hydroxyaspergillic acid.

Muta-aspergillic acid was converted to 2,5-diketopiperazine compound $C_{11}H_{20}N_2O_2$, (V) through monobromo compound, m.p. 113°C, (IV) by the route shown in the following figure, and this compound (V) was degraded to leucine and valine by hydrolysis with hydrochloric acid.

These results provided evidence that muta-aspergillic acid has the two side chain, isobutyl and isopropyl in its molecule.

Group analyses show the presence of one hydroxy (mono acetyl derivative, m.p. 111°C) and one n-hydroxy (Cu-salt, m.p. 231°C and $FeCl_3$ reaction).

Infrared spectrum of this antibiotic shows the same absorption band, at 1120^{-1} cm, as hydroxyaspergillic acid. It has been well established, in general, that tertiary alcohol has a C-O band at about 1150^{-1} cm and this band is shifted about -30^{-1} cm to longer wave length when α carbon to tertiary alcohol has the branch.

Moreover, as descrived above, this antibiotic is optically inactive and does not form any iodoform on treatment with hypoiodite.

These facts provide evidence that the hydroxy group of this antibiotic is tertiary and locates at the isopropyl side chaine.

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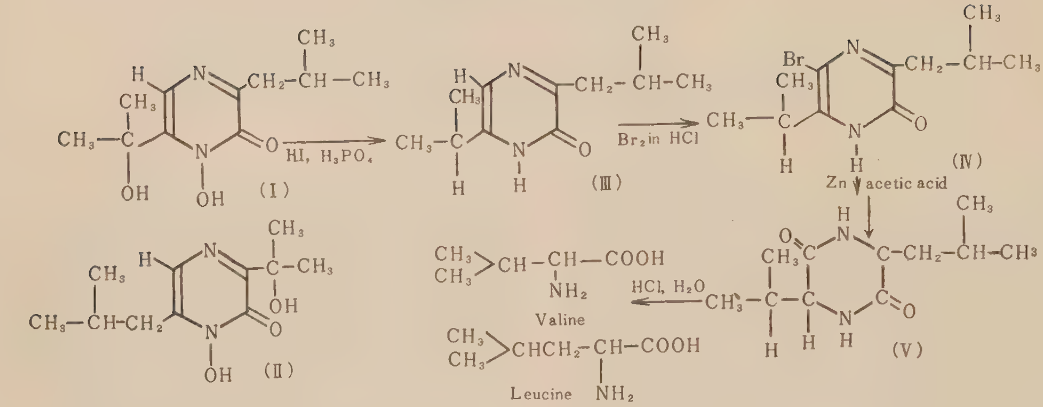
1) S. Nakamura and T. Shiro, *Bull. Agr. Chem. Soc. Japan*, **23**, 65 (1959).

2) J.D. Dutcher, *J. Biol. Chem.*, **171**, 321, 341 (1947); **232**, 785 (1958).

3) G. Dunn, J.J. Gallagher, G.T. Newbold and F.S. Spring, *J. Chem. Soc.*, S. 126 (1949).

4) G. Dunn, G.T. Newbold and F.S. Spring, *J. Chem. Soc.*, S. 131 (1949); 2679 (1951).

5) U. Weiss, F. Strelitz, H. Flow and I.N. Asheshov, *Arch. Biophys.*, **74**, 150 (1959).



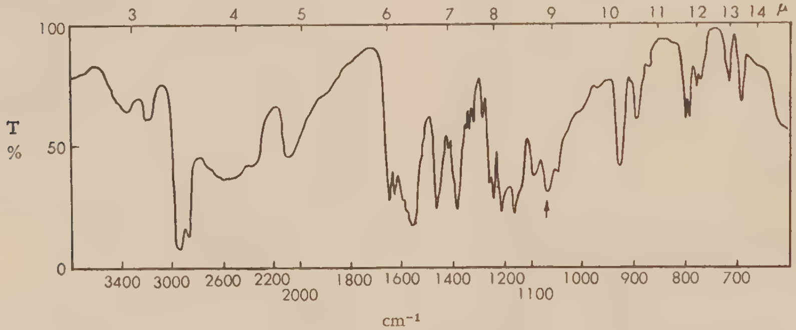


FIG. 1. Infrared Spectrum of Muta-aspergillic Acid (Nujol mull)

The above mentioned results lead to draw the conclusion that formula (I) or (II) is the most possible structure for this antibiotic.

The minimum concentration for complete inhibition of growth in a diluted Sake-peptone medium (composed of 70 parts of Sake, 30 parts of water and 1 part of polypeptone) against true hiochi-bacilli (*Lactobacillus homohiochii* H-42 and *Lactobacillus heterohiochii* H-1) was 5~10 meg/ml, and that against hiochilactobacilli (H-7 and H-34) 10~20 meg/ml.

At higher concentrations, it did not inhibit the growth of *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus bulgaris*, *Mycobacterium phlei*, Mycobacterium 607, *Penicillium chrysogenum*, *Aspergillus niger*, *Candida albicans*, *Saccharomyces ceroviciae*, *Tolura ntilis*, *Corynebacterium xerosis*.

LD_{50} for mice administrated intraperitoneally was 100 mg per Kg of body weight.

The author expresses his sincere thanks to Prof. Y. Sumiki, University of Tokyo for his guidance and encouragement throughout the course of this work and to Emeritus Prof. K. Sakaguchi and Prof. H. Umezawa for their sound advices and also to Mr. T. Shiro, Yamamura Sake-brewing laboratory and Mr. Y. Kurimura, Central Research Laboratory Sanraku Distillers Co. LTD. for their cordial assistances in this work.

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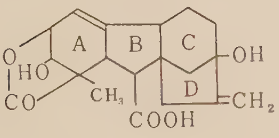
Biochemical Studies on "Bakanae" Fungus. Part 55

Synthesis of Gibberellins. Part I.
Infrared Spectra of the Lactones of Cyclohexane Series

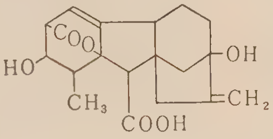
Sir:

Chemical structure of gibberellins has recently been elucidated to a great extent and formulas were assigned by Japanese and British workers which differ slightly each other. Cross et al.[1,2] had assigned (I) for gibberellin A_3, while (II) was assigned for it in the previous papers of this series[3,4]. The British workers revised the formula[5] and the absolute configuration of the revised formula was also determined[6] (III).

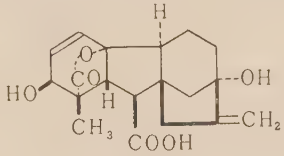
As can be seen from the formulas, the differences lie in the structure of ring A. Infrared absorption of lactone carbonyl of gibberellins occurs at 1750~1770 (Nujol mull) or at 1777~1786 (dioxan) cm^{-1}, which was attributed to γ-lactone by Cross et al., while strained δ-lactone structure was assigned by the Japanese group.



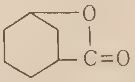
(I)



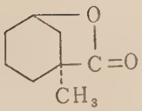
(II)



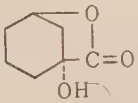
(III)



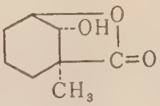
(IV)



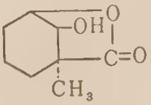
(V)



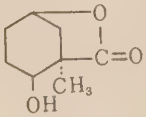
(VI)



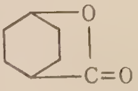
(VII)



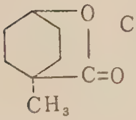
(VIII)



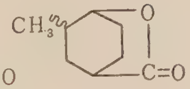
(IX)



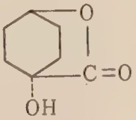
(X)



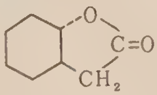
(XI)



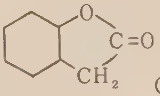
(XII)



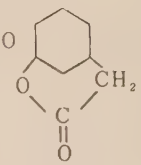
(XIII)



(XIV)



(XV)



(XVI)

In order to clarify this point, reliable data were necessary concerning the infrared frequencies of the lactones of cyclohexane series which were obtained by syntheses. Therefore we prepared 13 lactones (IV)~(XVI) and examined their infrared spectra. Among them,

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1) B.E. Cross, J.F. Grove, J. MacMillan and T.P.C. Mulholland, *Chem. and Ind.*, **36**, 954 (1956).

2) B.E. Cross, J.F. Grove, J. MacMillan, T.P.C. Mulholland and N. Sheppard, *Proc. Chem. Soc.*, **1958**, 221.

3) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **22**, 432 (1958).

4) N. Takahashi, Y. Seta, H. Kitamura and Y. Sumski, *ibid.*, **23**, 509 (1959).

5) B.E. Cross, J.F. Grove, J. MacMillan, J.S. Moffatt, T.P.C. Mulholland, J. Seaton and N. Sheppard, *Proc. Chem. Soc.*, **1959**, 302.

6) B.E. Cross, P. McCloskey, T.P.C. Mulholland and W. Klyne, *Chem. and Ind.*, **1959**, 1345.

TABLE I. POSITION OF CARBONYL STRETCHING BAND OF THE LACTONES PREPARED

Name of the Compd.	m.p. or (b.p.)	Wave Number (cm^{-1})	
		Nujol mull	Dioxan
A. γ-Lactones of Cyclohexanolcarboxylic Acids			
Cyclohexanol-(3)-carboxylic acid lactone (IV)	118~120°C	1770	1778
1-Methylcyclohexanol-(3)-carboxylic acid lactone (V)	34~35°C	1768	1775
Cyclohexane-*trans*-diol-(1, 3)-carboxylic acid lactone (VI)	165~165.5°C	1755	1782
acetate	72~74°C	1790	—
tosylate	135~136°C	1792	—
1-Methylcyclohexane-*trans*-diol-(2, 3)-carboxylic acid lactone (VII)	72~73°C	1745	1780
phenylurethan	143~144°C	1780	—
1-Methylcyclohexane-*cis*-diol-(2, 3)-carboxylic acid lactone (VIII)	(173~177°C/10 mm)	1768 (broad) (L)*	1778
phenylurethan	129~130°C	1778	—
1-Methylcyclohexane-*cis*(?)-diol-(2, 5)-carboxylic acid lactone (IX)	101~101.5°C	1742	1778
tosylate	156.5~157°C	1772	—
B. δ-Lactones of Cyclohexanolcarboxylic Acids			
Cyclohexanol-(4)-carboxylic acid lactone (X)	108~110°C	1740	1752
1-Methylcyclohexanol-(4)-carboxylic acid lactone (XI)	54.5~56.5°C	1742	1750
3-Methylcyclohexanol-(4)-carboxylic acid lactone (XII)	46~47°C	1740	1752
Cyclohexane-*trans*-diol-(1, 4)-carboxylic acid lactone (XIII)	152~153°C	1745	1762
acetate	84~85°C	1766	—
tosylate	129~130°C	1768	—
C. Lactones of Cyclohexanolacetic Acids			
trans-Cyclohexanol-(2)-acetic acid lactone (XIV)	(104°C/4 mm)	1778 (L)	1780
cis(?)-Cyclohexanol-(2)-acetic acid lactone (XV)	(120°C/9 mm)	1780 (1754 sh.)	1780
Cyclohexanol-(3)-acetic acid lactone (XVI)	124~125°C	1730 (L)	1730

* "L" means the values determined in the states of liqued films.

(VII) corresponds to ring A of gibberellin A_1 of Cross' first formula, and (IX) to that of the revised formula, respectively.

Infrared frequencies of carbonyl groups of lactones and their derivatives are shown in Table I.

Carbonyl streching band of γ-lactones occurs in the range 1742~1780 cm^{-1} (Nujol mull or liquid film). A considerable shift toward lower frequencies is observed in the lactones with a free hydroxyl group. However, even in these hydroxy-lactones, absorption occurs at 1772~1792 cm^{-1} (Nujol mull) if the free hydroxyl is converted into acetate, tosylate or phenylurethan. All γ-lactones, with or without a free hydroxyl, absorb at 1775~1782 cm^{-1} in dioxan solution.

δ-Lactones absorb in the range 1730~1745 cm^{-1} (Nujol mull or liquid film). Tosylation or acetylation of a hydroxy-lactone causes a shift toward higher frequencies.

Thus we are led to the following conclusions:

(i) γ-Lactones generally absorb at higher frequencies than δ-ones, but there are some which absorb at nearly the same frequencies as δ-ones owing to the interaction of free hydroxyl with lactone carbonyl.

(ii) γ-Lactones can easily be distinguished from δ-ones by examining their infrared spectra in dioxan solutions, since the dipolar interactions are nearly eliminated in that solution.

(iii) From these results, the lactone ring in gibberellins seems to be γ.

Details will soon be published elsewhere.

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Formation of L-Glutamic Acid from Fumaric Acid by Bacteria. Part III. Condition of Culture.

(p. *531～535*)

By Ryohei AOKI, Yasuhiro KONDO, Toshinao TSUNODA and Tetsuo OGAWA

(*The Central Research Laboratory of Ajinomoto Co. Inc.*)

The authors have made studies on some suitable culture conditions for glutamate production by *Bacillus pumilus*. The glutamate was determined by using medium containing various ratios of glucose to fumaric acid, and calculation was made on the presumption that the glutamate is synthesized from fumarate via oxalacetate. It was revealed that the formation of oxalacetate is mainly due to the conversion of fumarate.

Among TCA-cycle members, fumarate was found to be the best carbon source for glutamate production, succinate and α-ketoglutarate following, whereas malate and citrate were converted to only a small amount of glutamate. The yield of glutamate could not be increased by pH control under standard culture condition.

Among the culture conditions tested, e. g., heat shocking, seed volume and the effects of some growth factors, heat shock treatment coused on increase in the amount of glutamate.

Formation of L-Glutamic Acid from Fumaric Acid by Bacteria. Part IV. Chemical Changes in Culture and Enzymatic Activity.

(p. *536～540*)

By Ryohei AOKI, Yasuhiro KONDO, Toshinao TSUNODA and Tetsuo OGAWA

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Chemical changes occurring during culture and the enzymatic activity of *Bacillus pumilus* were studied.

Quantitative analysis of amino acids and organic acids in the culture broth was carried out by applying ion exchange chromatography, paper chromatography paper other methods.

As for the nitrogen balance in the final broth, organic nitrogen was determined as approximately 60% of total nitrogen. Glutamate, alanine, aspartate, pyroglutamate and cellular nitrogen were determined as 70, 10, 5, 6 and 6% of organic nitrogen, respectively. As for the carbon balance, amino acids, organic acids, glycerol and cellular carbon were determined as 58, 5, trace, trace and 3% of organic carbon, respectively. Lactate and volatile acids were the main products of organic acids, and ketonic acids, succinate, lactate and fumarate were observed in small quantities. When washed cells of *B. Pumilus* were incubated in a medium containing potassium glutamate, no decrease in the potassium glutamate content was observed.

The oxygen uptake of potassium glutamate, α-ketoglutarate, succinate and fumarate was determined with dry-cells. Likewise glutamic decarboxylase, aspartic-glutamic transaminase, aspartic-alanine transaminase, and aspartases I and II were determined with the dry cells.

As a matter of fact, the aspartases and aspartic-glutamic transaminase were found to be relatively more active than the others, and these enzymes are considered to be responsible for the fermentation process of glutamate production from fumarate by employing *B. pumilus*.

Studies on the Fermentation Products of *Clostridium aurantiacum.*

(p. *540～545*)

By Yasunosuke NAMBA and Chôseki FURUSAKA

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Kenji KURASHIMA and Tsuneji SUTO

(*Faculty of Agriculture, Tohoku University, Sendai*)

Clostridium aurantiacum strain O which produce orange-red viscus substance and strain O-1 which produce white one always produced a large number of butanol as the main fermentation product when various carbon and nitrogen sources were used. The analysis of solvents produced by Johnson's method showed the following results (bacteria are grown Starkey's medium containing peptone as a nitrogen source); Butanol: Ethanol: Acetone: iso-Propanol, 4.73: 2.73: 0.08: 0.73 mg/ml, respectively. The result by the gas liquid chromatographical technique was 66.5: 7.4: 0.5: 25.6 per cent, respectively, and each solvent ratios were 56.5: 32.7: 1.2: 9.6 per cent, respectively, by Johnson's method.

Those bacteria both the acids forming period and the acids reducing period along the development of their growth as in the case of the solvent producing *Clostridium*, but the acids forming period is not clearly explicit.

Those bacteria are noted with active acetoacetate decarboxylase, despite of the fact that the accumulation of acetone is not remarkable in their fementration.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part I. Effects of Temperature, Air and Light on the Color Reversion of Refined Soybean Oil, and Method of Measuring Color Reversion.

(p. *545~551*)

By Ichiro HARADA, Yutaka SARATANI and Masao ISHIKAWA

(*Shimizu Plant, Hohnen Oil Co., Ltd.*)

Color development observed mainly on refined soybean oil during storage was named color reversion. Some factors affecting the rate of color reversion oil were examined.

The rate of color reversion of deodorized oil increased with temperature, and after reaching the maximum color value the colored oil was bleached.

The rate of bleaching also increased with temperature.

The rate of color reversion of deodorized oil depends on oxygen pressure and under reduced atmospheric pressure (less than 5mm Hg) the color of oil remained unchanged.

When the refined soybean oil was irradiated intermittently with sunlight, it took place reversible photo-bleaching phenomena. The mechanism of this phenomena is connected with photo-oxidation of fat at the presence of trace chlorophyll.

The results obtanied suggest that color reversion and autoxidation of fat belong to the same reaction system.

The method of accelerating color reversion in a short time was determined with reference to above factors.

Studies on the Color Reversion of Refind and Deodorized Soybean Oil. Part II. General Aspects of Color Reversion, and Relations between Color Reversion and Tocopherol Contents.

(p. *551~558*)

By Ichiro HARADA, Yutaka SARATANI and Masao ISHIKAWA.

(*Shimizu Plant, Hohnen Oil Co., Ltd.*)

Among the vegetable oils, soyobean oil showed most remarkable reversion of color, but when the absorption spectra of fresh oil and colored oil were compared no apparent change was observed in the conjugated double bond of fatty molecule.

It appears that the substrate of color reversion exists in the unsaponifiable matters of soybean oil.

On soybean oil, only the deodorized oil showed reversion of color and undeodrized oil did not. It seems that this nature is given by the effect of heat in deodorization.

It was found that the substrate of reversion was stable to alkali treatment and had weak absorpability to activated clay, strong resistance to heating, thus it was distillable by deodorization.

It was also found that the peak value of color reversion was parllel to the tocopherol content of oil before reversion.

These results demonstrate the intimate relations between the substrate of this reaction and tocopherol.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part III. Influences of Iron and Antioxidants on Color Reversion.

(p. *558~562*)

By Ichiro HARADA, Yutaka SARATANI and Masao ISHIKAWA.

(*Shimizu Plant, Hohnen Oil Co., Ltd.*)

When soybean oil was heated, the presence of metalic iron markedly increased the rate of color reversion of refined soybean oil and catalytic effect of iron was increased in proportion to temperature.

Evidences were obtained to indicate that iron in soybean oil was complexed with peroxide of fat, and by heat treatment complex was decomposed to active radical and iron, and the reaction of color reversion was accelerated.

Color reversion of soybean oil was not inhibited by addition of primary antioxidants, but it was retarded by addition of synergists. This retarding effect probably attributes to the action of synergist as a metal-inactivator. Such chelating agent will react only with free metal and not with metal-hydroperoxide complex.

On the Movement of Barium Sulfate and Chromic Oxide in the Large Intestine. (On the Methods for the Study of Digestion and Absorption. Part III.)

(p. *563~566*)

By Ichiro YOSHIHARA, Akio SUGISAKI (*Faculty of Agriculture, University of Niigata*) and Makoto KANDATSU (*Faculty of Agriculture, University of Tokyo*)

A Mixture of ^{35}S-barium sulfate and chromic oxide was injected into the cecum of the rabbit with abdominal window. Radioactivity, chromic oxide and barium sulfate in ash of feces were assayed.

C. P. M. : Cr_2O_3 ratio per 100 mg of fecal ash was different in the samples of feces showing a regular tendency and it was confirmed that the tendency was based upon tne compositional differences in ash of feces.

A sure evidence of the parallel movement of barium sulfate and chromic oxide in the large intestine was noticed.

Studies on Shinshiki Shoyu (Semi-Chemical Soy Sauce). Part IV. Isolation and Identification of the Volatile Sulfur Components. (p. *566~569*)

By Takahiro UENO and Akio NOBUHARA

(*Noda Soy Sauce Co. Ltd.*)

In order to recover the volatile sulfur components in Semi-chemical and chemical soy sauce, the exhaust gases from the aerated neutralysate of defatted soybean hydrolysate were passed through a train of absorption traps which consisted of anhydrous calcium chloride, lead acetate, 4% mercuric cyanide (aq.) and 3% mercuric chloride (aq.). 3 l of the neutralysate were boiled under reflux and aerated with nitrogen for 10 hours at the rate of 200 ml/min. by applying a vacuum at the end of the train.

Hydrogen sulfide was identified as lead sulfide in the lead acetate trap. From the white precipitate appeared in the mercuric cyanide trap, methyl mercaptan was identified as mercury methyl mercaptide. From the white precipitate appeared in the mercuric chloride trap. Dimethyl sulfide was identified as mercury salt of dimethyl sulfide and the presence of a trace of dimethyl disulfide was presumed. The characteristic flavour formed by acid hydrolysis is generally attributed to a complex mixture of these volatile sulfur compounds, carbonyls, acidic substances and etc.

Studies on Shinshiki Shoyu (Semi-Chemical Soy Sauce). Part V. Some Factors Affecting the Occurence of Volatile Sulfur Compounds. (p. *569~572*)

By Takahiro UENO and Akio NOBUHARA

(*Noda Soy Sauce Co. Ltd.*)

1) Some factors affecting the occurence of volatile sulfur compounds under the experimental procedure, described in the previous papers were investigated. Below 85°C the occurence of these volatile sulfur compounds was only a little, but at 100°C much larger amounts of volatile sulfur compounds were evolved, especially dimethyl sulfide. The occurence of hydrogen sulfide and methyl mercaptan increased in proportion to the heating time. But that of dimethyl sulfide became almost constant in 2~4 hours after boiling.

Hydrogen sulfide was more readily evolved in strong acidic solution and diminished in proportion to pH ascending. But above about pH 5, increased gradually. Methyl mercaptan and dimethyl sulfide occured only a little in strong acidic solution and increased remarkably in proportion to pH ascending

2) The occurence of these volatile sulfur compounds in various soy sauce was compared.

In the occurence of hydrogen sulfide and methyl mercaptan, there were no differences between naturally brewed soy sauce and chemical soy sauce. In the case of dimethyl sulfide, naturally brewed soy sauce evolved only a trace of it but chemical soy sauce evolved much larger amounts of it.

Dimethyl sulfide is one of a chracteristic consituent of soy sauce which underwent acid hydrolysis.

Movement of Chlorine in the Rice Kernel. Part III. Moisture in Rice Kernel as the Dominant Factor for the Chlorine Translocation.

By Shoji KUBO and Kyoko FUJITA (*Food Research Institute, Ministry of Agriculture and Forestry*)

(p. *572~576*)

30 sorts of brown rice (5 varieties each cultivated in 6 districts in Japan) were placed under 10 circumstances of various combinations of temperature and humidity, and the translocation of chlorine in each rice kernel was histochemically checked time to time for 10 months.

For all 30 samples, the translocation occurred first under a condition of high temperature and humidity, and second at low temperature and high humidity. The movement of chlorine was not observed at all under 7 conditions of lower humidity.

It seemed very probable that a certain amount of "free water" was necessary for the chlorine translocation, and the practical limit of the water content to have chlorine move into endosperm was supposed as 14.5~15% at 22°C, or 15.5~16% at 5°C.

A species "Aikoku" was different from the other 4, that its chlorine moved very easily, neverthless water content of the species was 0.2~0.5% lower than the others in each circumstance. Some differences of speed or time of chlorine translocation were observed between the samples from 6 districts.

Movement of Chlorine in the Rice Kernel. Part IV. Relations of the Inner Translocation of Chlorine with the Increasing of Inorganic Phosphorus and the Change in Germination Power.

By Shoji KUBO and Kyoko FUJITA (*Food Reserach Institute, Ministry of Agriculture and Forestry*)

(p. *577~579*)

The contents of inorganic phosphorus were determined on each sample of the preceeding report (part III), after 10 months of storing period, and the relation with their rates of chlorine translocation was followed. The changes in germination power were also compared from the same viewpoint.

The increase of inorganic phosphorus and the decrease of germination power at lower temperature (13~15°C) seemed to have a clear parallelism. But they showed no practical correlation with the rate of chlorine translocation. It was rather interesting that samples stored in a condition of high temperature and the lowest humidity, which had a higher content of inorganic phosphorus, showed a stronger germination than those stored under lower temperature of high or low humidity.

The results seemed to encourage the opinion of the authors that chlorine translocation in the rice kernel depended on the contents of "free water" in the rice as a dominant factor.

Researches on Ergot Fungus. Part XXXVIII.

On the Saprophytic Production of Ergot Alkaloids by *Claviceps purpurea* (Fr.) Tul. (p. *580~584*)

By Matazō ABE, Tōgo YAMANO, Saburō YAMATODANI, Yoshiharu KŌZU, Mitsugi KUSUMOTO, Hajime KOMATSU and Saburō YAMADA.

(*The Institute for Fermentation, Osaka*)

The authors have reported on the formation of ergot alkaloids in the saprophytic culture (surface technique) of a *Claviceps purpurea* strain obtained from the Spanish ergot of rye.

They have succeeded in isolating the following alkaloids from the mycelium (750 mg, based on dry weight) and the culture filtrate (45 l): peptide-type alkaloids (ca. 1.3g yield), ergometrine (45mg), ergometrinine (96mg), agroclavine (32 mg), secaclavine (5 mg) and a new water-soluble alkaloid (20 mg). The new water-soluble alkaloid was isolated together with other alkaloids, also, from the said Spanish ergot itself in almost the same yield. This new alkaloid, $C_{24}H_{28}O_4N_4$, m.p. 217°C (uncorr. decomp.), $[\alpha]_D^{18} = +298°$ (c. 0.2 in $CHCl_3$), $[\alpha]_D^{18} = +417°$ (c. 0.22 in pyridine), was presumed to be a special peptide-type alkaloid having isolysergic acid nucleus in its molecule, and was named ergosecalinine. Ergosecalinine yielded pyruvic acid together with lysergic acid on alkaline hydrolysis, while on acid hydrolysis it afforded only one amino acid, valine. When boiled in a mixture of ethanol and acetone containing phosphoric acid, regosecalinine was converted into an unknown substance which was nothing but the alkaloid, ergosecaline. All attempts to obtain ergosecaline pure were unsuccessful despite various experiments.

Part of this paper was communicated in *Bull. Agr. Chem. Soc. Japan*, **23**, 246 (1959).

Researches on Ergot Fungus. Part XXXIX.

On the Method for Systematic Extraction of the Clavine-Series Ergot Alkaloids. (p. *584~589*)

By Saburō YAMATODANI

(*The Institute for Fermentation, Osaka*)

1) Partition ratio of several Clavine-series ergot alkaloids produced by *Elymus*-type or *Agropyrum*-type ergot fungus between chloroform, benzene or ethylacetate and various McIlvaine's buffers has been determined. From the pH-partition curves obtained, the author has established a fundamental method of separating those alkaloids into individuals or groups by systematic extraction.

2) In the course of the present work, a Clavine-series ergot alkaloid, costaclavine, was found and isolated in crystalline form, also, from the saprophytic culture of *Elymus*-type ergot fungus.

3) Two Clavine-series ergot alkaloids, festuclavine and pyroclavine, were difficult to separate from each other by the above method, but they were clearly separated by a certain alumina column chromatography.

Studies on L-Glutamic Acid Fermentation. Part III.

Distribution and Activity of L-Glutamic Acid Dehydrogenase in Microorganisms. (p. *589~593*)

By Shukuo KINOSHITA, Katsunobu TANAK and Sadao AKITA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. Co., Ltd.*)

Among bacteria tested, strong activity of L-glutamic acid dehydrogenase was observed in *Micrococcus glutamicus* (a potent glutamic acid producer from glucose and ammonia), *Proteus vulgaris*, *Pseudomonas ovalis* and *Serratia macerans*, whilst activity of the enzyme was usually low in *Bacilli* and *Microccoci* (with exception of *Micrococcus glutamicus*).

It was also noted that, in bacteria, the glutamic acid dehydrogenase activities was quite variable between genera, spiecies and strains, but yeast and fungi generally have high activities of the enzyme.

It is probable that the high efficiency of glutamate synthesis in aerobic condition by *Micrococcus glutamicus* is due to the presence of high glutamic acid dehydrogenase activity and the impairment of one or more component of TPNH oxidase system.

Studies on L-Glutamic Acid Fermentation. Part V.

Biotin and L-Glutamic Acid Accumulation by Bacteria. (p. *593~600*)

By Katsunobu TANAKA, Takeshi IWASAKI and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. Co. Ltd.*)

Effect of various components of medium on fermentation products by *Micrococcus glutamics* was investigated using synthetic media. Experimental results showed that biotin had a very important role in shifting fermentation products in this organism. Especially, for the production of L-glutamate, the biotin concentration of the medium is of critical importance. In limiting concentration of biotin, a large amount of L-glutamate and small amount of α-ketoglutrate and lactate were accumulated. On the other hand, in excess of biotin, heavy cell growth and rapid utilization of glucose took place and they were accompanied by accumulation of lactate as a main product. In the latter case, significant increase of succinate, acetate and alanine was observed.

It was also observed that other biotin requiring microorganisms, i. e., *Bacillus circulans* and *Bacillus lentus* accumulated a large amount of L-glutamate from glucose and ammonium sources only in media with limited supply of biotin.

It is almost sure that restriction of the growth of these microorganisms due to the limitted supply of biotin results in impairment of one or mere biotin dependent metabolic reactions, and that this impairment is accompanied with an accumulation of L-glutamate.

Studies on L-Glutamic Acid Fermentation. Part VI. The Role of Biotin in Metabolism of *Micrococcus glutamicus*. (p. *600～608*)

By Katsunobu TANAKA, Sadao AKITA, Kazuo KIMURA, and Shukuo KINOSHITA

(*Tokyo Research Laboratory, Kyowa Fermentaion Industry Co., Ltd.*)

The role of biotin in a number of enzyme systems of *Micrococcus glutamicus* has been investigated using normal and biotin defficent cells in order to clarify the mechanism of potent glutamate accumulation shown by this organism grown in biotin limited media.

From the experimental results, biotin seems to have a marked effect on glycolises (may be hexokinase), succinate oxidation system, α-ketoglutarate oxidation system, aspartase, and glutamate utilization which are possibly to have an important role in the shift of fermentation products by the organism.

It seems that biotin has a key role for the carbohydrate metabolism to proceed either to TCA cycle or to other paths (DCA cycle or glyoxylate cycle), and also control the ratio of biosynthetic rates of glutamate and aspartate.

These findings were discussed in conection with the mechanism of glutamate accumulation and carbohydrate metabolism of this organism previously reported.

Studies on the Organism Producing Isopropanol from Acetone. Part V. Enzymological on the Oxidation-Reduction of *Lactobacillus brevis* var. *hofuensis*. (p. *608～615*)

By Kazuo HOSHINO

(*Kyowa Fermentation Industry Co. Ltd.*,)

Two oxidation-reduction systems of isopropanol-acetone and ethanol-acetaldehyde were investigated about 02-uptake, dye reduction and redox of TPN. The crude cell-free extracts of *L. brevis* var. *hofuensis* were prepared by sonic oscillation and SiO_2-grinding. It has been visualised that the latter extract contains more enzymes for isopropanol-acetone system, however, the substrate specificity for O_2-uptake and reduction by TPNH exhibits opposite feature about two systems. Isopropanol is oxidized more rapidly than ethanol in O_2-uptake or dye (2,6-dichlophenol-indophenol) reduction, while acetone is reduced less than acetaldehyde by TPNH. It has been also demonstrated that the flavoprotein like cytochrome reductase participates between the dehydrogenation and the respiration as the results of the experiments of the consumption of TPNH by cell-free extract and the cyanide inhibition against O_2-uptake. This flavoprotein does not link to mammalian cytochrome c. From inhibitors experiments it is also demonstrated that *p*-chloromercuribenzoate inhibits the reduction of acetaldehyde, but not acetone reduction. Furthermore, iodoacetic acid does not inhibit the reduction of acetaldehyde and acetone. From these findings the author has concluded that the enzyme concerning the oxidation-reduction between isopropanol and acetone is different from alcohol dehydrogenase and supposed that the participation of flavoprotein to the redox system causes the shift of the oxidation-reduction potential and results in the rapid oxidation of isopropanol. The author has proposed the name of isopropanol dehydrogenase to the enzyme concerning the oxidation-reduction between isopropanol and acetone.

Studies on the Organism Producing Isopropanol from Acetone. Part VI. Isopropanol Dehydrogenase and Alcohol Dehydrogenase of *Lactobacillus brevis* var. *hofuensis*. (p. *616～619*)

By Kazuo HOSHINO

(*Kyowa Fermentation Industry Co. Ltd.*,)

The purification has been carried out about isopropanol dehydrogenase and alcohol dehydrogenase of *L. brevis* var. *hofuensis*. The activity increases about 5-folds for isopropanol dehydrogenase and 40-folds for alcohol dehydrogenase. The substrate specificity of isopropanol dehydrogenase has been observed about various alcohols and diols. The specificities of isopropanol, 1,2 propylene glycol and *sec*-butanol were increased with the purification of isopropanol dehydrogenase. It has been demonstrated that the purified preparation of isopropanol dehydrogenase shows the specific absorption spectra of flavoprotein, and that the peaks of dfference spectrum are observed at 380 mμ and 44mμ. From these results the author has supposed that the linkage of isopropanol dehydrogenase and flavoprotein results in the shift of equilibrium and potential of isopropanol-acetone system and favours the oxidation of isopropanol in the cell-free extract of this organism.

A Simple Quick Method for Quantitative Determination of Amino Acids by Paper Chromatography.

(p. *620～624*)

By Yasushi WATANABE, Kikuko WATANABE, Fusaoki KOIDE, Tadashi SAITO and Kensuke SHIMURA

(*Laboratory of Biochemistry, Department of Agriculture, Tohoku University*)

A simple quick method for quantitative determination of amino acids by paper chromatography was developed. A sample solution containing 10γ to 40γ of amino acids was adjusted to neutral pH, and applied to a Toyo filter paper No. 50 (40×40cm) in 2 cm width. After the chromatograpy has been run in a chamber, the paper were dried, and then sprayed with 0.2% ninhydrin in acetone. The color was developed by heating for 3 minutes at 60°C. 2% ninhydrin in acetone was infiltrated into weekly developed zone by pipet, and the paper was dried in an oven for 3 minutes at 60°C. After the zone was sprayed sufficiently with 1/25 M phosphate buffer pH 7.0, the color was developed again by heating for 40 minutes at 60°C.

The colored zones were cut into a strip, heated with steam for 90 seconds and then the spots of each amino acid were cut off. The color was extracted with 5 ml of methanol containing equal volume of 1/25 M phosphate bufier of pH 7.0. The color density was measured using a Hitachi photoelectric colorimeter with 570 mμ filter.

The present method is suitable for the assay of amino acids in sample solutions of relatively simple amino acids composition such as the solutions from enzymatic reaction, amino acid fermentation, etc.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKUKAISHI
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Studies on the Fermented Milk. Part VI. On the Non-fermentable Milk. Part I. The survey of Inhibitory Substances in Lactic Acid Fermentation (p. *625∼629*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co., Ltd.*)

Non-fermentable milk was found in a raw milk supply for the sour milk drink preparation. This milk does not take normal lactic acid fermentation. By the survey of inhibitory substances, it was made clear that the small amount of penicillin in milk inhibit the normal fermentation by *Lactobacillis bulgaricus*.

By this successive works, the followings were induced that the heat-stability of penicillin in milk is very high and penicillin is not inactivated by heating at 120°C for 30 min. and that *L. bulgaricus* is highly sensitive for penicillin.

Studies on the Adsorption Indicator in the Determination of Sodium Chloride in Soy-Sauce and Misco. Part I. Argentometry by the Use of Bromophenol Blue as an Adsorption Indicator (p. *630∼634*)

By Kenji SATO

(*Brewing Experiment Station, Tax Administration Agency*)

A rapid accurate determination of chloride in Soy-Sauce and Miso is very difficult. Mohr's method is unsuitable, because (I) organic substances in Soy-Sauce and Miso impede the color change of reagents (II) the color of Soy-Sauce and Miso resembles that of Ag_2CrO_4, and the results indicate high values of 2∼4%. Volhard's method is also unsuitable. Investigating on direct argentometry of Soy-Sauce and Miso, obtained the following results.

(1) In the titration of Cl^- in Soy-Sauce and Miso with $AgNO_3$, satisfactory results are obtained in acedic solution (optimum pH: 2.7∼3.4) with bromophenol blue as an adsorption indicator. Deproteinization of the fluids is not necessary. The results are accurate in the presence of considerable quantities of protein, amino acids and other organic compounds.

(2) Metanil yellow, tropaeolin O.O., fluorescein, dichlorofluorescein, bromophenol blue, congo red etc. cannot be used owing to the interference of proteins and their hydrolyzid or fermented products.

Studies on the Adsorption Indicator in the Determination of Sodium Chloride in Soy-Sauce and Miso. Part II. Argentometric Determination by the Use of Some Sulfonphthalein Dyes as Adsorption Indicators. (p. *634∼637*)

By Kenji SATO

(*Brewing Experiment Station, Tax Administration Agency*)

(1) Chlorophenol blue (I) and chlorobromophenol blue (II) are new argentometric adsorption indicators for titrating Cl^-, Br^- and I^-. These indicators are reversible and the color changes at the end points are from yellow to blue in all cases. The titration is possible up to a concentration of 0.02NCl^-, Br^-, I^- with I and 0.02NCl^-, 0.01NCl^-, 0.01N Br^-, 0.01N I^- with II in a weakly acedic solution. (pH: 3.0∼4.0)

In the volumetric analysis of chlorides in Soy-Sauce and Miso with $AgNO_3$, testing of 13 kinds of sulfonphthalein indicators, titration is successful with tetrabromophenol blue as an adsorption indicator. The pH must be adjusted to 3.6∼2.8. In this case, P.R., C.R., T.B., B.P.R., B.C.P., C.P.R., I.P.B., C.P.B. and C.B.P.B. cannot be applicable owing to the protein's interference, and it was clarified theoretically from the view of chemical structure that bromophenol blue and tetrabromophenol blue are applicable to Soy-Sauce and Miso.

Studies on the Adsorption Indicator in the Determination of Sodium Chloride in Soy-Sauce and Miso. Part III. Argentometric Determination by the Use of Fe^{3+} (or Cl^{4+}, VO_3^-)–*o*-tolidine System as an oxiadsorption Indicator. (p. *638∼644*)

By Kenji SATO

(*Brewing Experiment Station, Tax Administration Agency*)

(1) In the argentometric determination of Cl^- in Soy-Sauce and Miso, diphenylamine, N–methyldiphenylamine red, benzidine, *o*-dianisidine, *o*-tolidine, methyl violet (or acid violet, crystal violet), brilliant green, malachit green are suitable as adsorption indicators. Among above indicators, Fe^{3+}–benzidine, Fe^{3+}–*o*–tolidine oxiadsorption indicators are excellent.

The Fe^{3+}–*o*–tolidine oxiadsorption indicator is recommended as more sharply sensitive then bromophenol blue in the argentometry of Cl^- in Soy-Sauce and Miso. When Cl^- is in excess, the tolidine-meriquinoid is adsorped by AgCl, giving a purple color. At the end point a yellow color is obtained very sharply. The titration with Fe^{3+}–*o*–tolidine is successful in solutions containing excess of protein and by using Cl^{4+} or VO$_3^-$-*o*–tolidine system, this system permits high concentration of acid H$_3$PO$_4$ up to 30%. The evalution of Cl$^-$ with Fe^{3+}–*o*–tolidine gives satisfactory results in solutions containing up to 0.003NCl$^-$, and in ethanol solvent as little as up to 0.001NCl$^-$, and showed acid resistance up to 2.7N, 0.8N and 0.07N H$_2$SO$_4$ in concentration for 0.1NCl$^-$, 0.02NCl$^-$ and 0.003NCl$^-$ solutions respectively.

(2) In strongly acedic solutions, malachite green, brilliant green can be used as new adsorption indicators for titrating Cl$^-$, Br$^-$ and I$^-$ with AgNO$_3$.

Nitrate Reductase of Fowl Liver. (VI). Enzymatic Oxidation of DPNH by Nitrates. (p. *644~649*)

By Hirohisa OMURA and Yutaka OSAJIMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

It was demonstrated that the enzymatic activity which catalizes the oxidation of DPNH by nitrate is contained in the tissue of fowl liver. For the enzymatic activity, FAD and phosphate were required, while TPNH and FMN were ineffective. The activity was considerably labile to heating and showed the maximal activity at 30°C and pH 7.7, but did not be inhibited by air, NaN$_3$ and monoiod acetic acid, and maintained even after lyophilyzing. These properties were quite different from those of nitrate reductase which has intimate connection with aldehyde oxidase. Furthermore, it was also assumed that the reducing system from nitrate to ammonia was contained in the enzyme solution.

Polymerization of 2-Vinylfuran by the γ-Irradiation. (p. *649~652*)

By Akira MISHINA

(*The Initiute of Scientific and Industrial Research, Osaka University*)

2-Vinylfuran, Synthesized from furfural via 2-furylacrylic acid was polymerized by γ-irradiation using Co^{60} source, and the results were compared with that obtained from Catalytic polymerization by $\alpha\alpha'$-azobisisobutyronitril.

At lower levels, total doses less than $10^5\gamma$, the yields of polymer were quite low as well as the catalytic polymerization. However, by increasing total dose, such as $10^7\gamma$, the yield increased to be approximately ten-times as compared with the level of $10^5\gamma$, and the product turned to gel indicating that a cross-linking structure would had been formed.

Studies on the Lytic Enzyme Produced by *Brevibacterium*. Part I. Production of the Lytic Substance. (p. *652~656*)

By Ken'ichiro TAKAYAMA, Kiyoshi UDAGAWA and Shigeo ABE

(*Tokyo Research Laboratory, Kyowa Fermentation Industry Co. Ltd.*)

The bacteria which lysed mycelia of *Aspergillus oryzae* were isolated. An investigation was undertaken on some microbiological properties and cultural conditions for one of these bacteria which had produced the lytic substance, the most stable and powerful.

The bacterium was named *Brevibacterium lyticum* sp. nov. by the authors.

Only when the bacterium was cultivated in the media containing the fungal mycelia, the formation of the lytic substance and simultaneous disappearance of the mycelia were observed.

Formation of the lytic substance is assumed to be extracellular and induced by the mycelia added.

The most favorable pH value of the media for the formation of this substance were found to be 7.5 to 8.5.

Studies on the Lytic Enzyme Produced by *Brevibacterium*. Part II. Properties of the Lytic Enzyme. (p. *656~658*)

By Ken'ichiro TAKAYAMA, Kiyoshi UDAGAWA and Shigeo ABE

(*Tokyo Research Laboratory, Kyowa Fermentation Industry Co. Ltd.*)

The properties of the lytic substance which was produced by *Brevibacterium lyticum* sp. nov. were investigated and the following results were obtained.

This substance was salted out at 50 to 70% saturation with ammonium sulfate and was not dialyzed. It was inactivated at standing for several hours at room temperature. The active substance for the lytic action was an enzyme with an optimum pH and temperature at 7.0 to 7.5 and 30° respectively.

The lytic action of the enzyme was active to the cells of *Aspergillus*, *Penicillium*, *Mucor*, *Streptomyces* and heat-treated yeasts, but not to *Rhizopus*. Generally, bacterial cells were rather stable against the enzyme action.

Though the cell wall preparation obtained from *Aspergillus oryzae* was not attacked by protease, amylase and lysozyme, this enzyme had a remarkable lytic action to both the cell wall preparation and intact cell of the organism.

The liberation of hexoses, amino acids and a small amount of hexosamines from these cell preparations of

Aspergillus oryzae was observed after the enzyme action.

Studies on the Chemical Constituents of the "Cherry Red" Tobacco Plant. Part I. The Alkaloids. (p. *659~661*)

By Susumu MATSUYAMA and Kenzo ISHITOYA

(*Okayama Tobacco Experiment Station, Japan Monopoly Corporation*)

The conversion of nicotine to nornicotine has been studied in the "cherry red" strains, selected from Bright Yellow variety of tobacco. This conversion was observable about 20 days after the topping and its rate increased with the progress of maturity in those strains. In the course of flue-curing, the conversion was further remarkable and it chiefly took place in the early stage suitable for enzymic reactions.

Studies on the Chemical Constituents of the "Cherry Red" Tobacco Plant. Part II. Chlorogenic Acid and Polyphenoloxidase. (p. *662~665*)

By Susumu MATSUYAMA and Kenzo ISHITOYA

(*Okayama Tobacco Experiment Station, Japan Monopoly Corporation*)

The changes in chlorogenic acid contents, polyphenoloxidase activities and respiration rates of leaf slices have been studied in the "cherry red" strains of Bright Yellow variety of tobacco. At the late stage of the maturity, the "cherry red" strains showed often characteristic reddish brown spots on the leaves and exhibited lower contents of chlorogenic acid, higher amounts of leaf-slice respiration and higher activities of polyphenoloxidase than those of a "red free" strain of the same variety. The contents of chlorogenic acid of the leaves without midstems increased at the last stage of flue-curing in both strains. The cherry red pigments appeared at the last stage of flue-curing. During the period, the leaf moisture level seemed to be unsuitable for enzymic reactions and little oxygen uptake of leaf slices took place, so the pigments might be possibly formed through some thermal reactions.

Studies on the Safrole Derivatives. Part II. The Reactions of Formaldehyde on Safrole and Isosafrole. (p. *666~668*)

By Siro SUGAWARA

(*Central Research Institute, Japan Monopoly Corporation*)

The author studied on the reaction of formaldehyde on safrole or isosafrole in acetic acid by using sulfuric acid as a condensation-agent.

Safrole gave *di*-(6′-safryl)-methane in a good yield as a product of Baeyer's reaction, while isosafrole gave not only 3, 4-methylenedioxy-6-propenyl-1-benzyl-acetate as a product of this reaction, but 4-(3′4′-methylendioxy phenyl)-5-methyl-*m*-dioxane as a product of Prins' reaction, diisosafrole and resinous matter as a polymerization products.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part IV. Mutual Relations among Peroxide Formation, Tocopherol Destruction and Color Development in Autoxidizing Soybean Oil. (p. *668~673*)

By Ichiro HARADA, Yutaka SARATANI, Masao ISHIKAWA

(*Shimizu Plant, Hohen Oil Co., Ltd.*)

The course of oxidation of the deodorized soybean oil containing a synergist was investigated from the standpoints of peroxide formation, tocopherol destruction and color deepening. It was shown that the induction period of tocopherol destruction agreed almost with that of peroxide formation, tocopherol loss and P.O.V. at the break point (soon after the induction periods) being about 10% and 30 respectively.

In the oxidation without aeration, the oil reached to the maximum color at the break point of peroxide curve and then was bleached parallel to the destruction of tocopherol. On the contrary in the accelerated aeration test the oil showed the rapid increase of coloration soon after the begining of tocopherol destruction and reached to the peak of coloration in a short time, and then faded.

These results indicate that the close relation exists between the color reversion and antioxidant action of tocopherol. It seems that the color reversion in oxidizing soybean oil is due to quinone derivatives of tocopherol and that in the oxidation of oil without aeration, the color reversion results chiefly from the oxidation of α-tocopherol, and also that in the aerating oxidation, it results from the stepwise oxidation of α and non-α tocopherol.

Studies on the Color Reversion of Refined and Deodorized Soybean Oil. Part V. Influences of Traces of Ingredients on the Autoxidation of Refined Soybean Oil. (p. *674~677*)

By Ichiro HARADA, Yutaka SARATANI, Masao ISHIKAWA

(*Shimizu Plant, Hohnen Oil Co., Ltd.*)

Color reversion of soybean oil is accelerated by traces of metals, Na-soap etc. The traces of these substances are partly extractable with water, hydrochloric acid or alkali, but it is difficult to remove them completely with ordinary extracting agents.

In the oxidation of deodorized soybean oil containing no synergist, iron added to the oil in the form of ferric sulphate destroyed the tocopherol vigorously, whereby rapid color development and peroxide formation were observed.

From the basis of these data, new method for following the rate of oxidation of vegetable oils by measuring the destruction rate of tocopherol was suggested in place of usual peroxide measurment.

Syntheses and Configurational Elucidations in the Rotenoid Series. Part XVI. Syntheses of Several New Compounds Related to Rotenoid (Coumarones, Coumarins). (p. *678~683*)

By Masateru MIYANO, Shigeru MURAKI, Toshihiko NISHIKUBO and Masanao MATSUI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

i) Several new coumarones and coumarins related to rotenone were synthesized.

ii) An anomalous selenium dioxide oxidation was found: 2-acetyl-4-hydroxy-5-carbomethoxy-coumarone when oxidized with equimolecular amount of selenium dioxide in an usual condition afforded 2-carboxy-4-hydroxy-5-carbomethoxy-coumaron in good yield.

iii) An anomalous azlactone synthesis was also found; the condensation of 2, 6-diacetoxy-benzaldehyde with hippuric acid in the presence of acetic anhydride and sodium acetate resulted in a single product which is 3-benzoylamino-5-acetoxy-coumarin instead of the expected azlactone.

iv) Certain Friedel-Crafts reactions of 4-methyl-7-hydroxy-coumarin gave 7-acyloxy compounds instead of 7-hydroxy-8-acyl compounds.

Syntheses and Configurational Elucidations in the Rotenoid Series. Part XVII. Syntheses of Several New Compounds Related to Rotenoid (Benzalacetones, *o*-Benzyloxy-phenylacetonitril, Chromans etc.). (p. *683~688*)

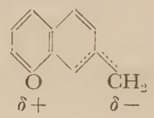
By Masateru MIYANO, Shigeru MURAKI, Tadashi KUSUNOKI, Takashi MORITA and Masanao MATSUI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

i) Several new 2, 6-disubstituted benzalacetones, chromenes and other compounds were synthesized and characterized.

ii) *o*-Benzyloxy-phenylacetonitril was prepared from salicylaldehyde through *o*-benzyloxy-benzaldehyde, *o*-benzyloxy-benzal-azlactone, *o*-benzyloxy-phenylpyruvic acid β-(*o*-benzyloxy-phenyl)-α-oximino-propionic acid.

iii) 2-Methyl-benzopyrylium chloride prepared by the condensation of salicylaldehyde, acetone and anhydrous hydrogen chloride when treated with equimolecular amount of sodium hydroxide afforded a compound which was determined to be 2-methylene-benzopyrylium. The latter is a quite new type compound and is supposed to have an aromatic character,



Movement of Chlorine in the Rice Kernel, Part V. Inner Translocation of a few Elements other than Chlorine (p. *689~694*)

By Shoji KUBO

(*Food Research Institute, Ministry of Agricuiture and Forestry*)

A detailed chart of inner distribution of the seven elements (K, P, Mg, Ca, Mn, Fe, Si) in the Japanese rice kernel was obtained by fractionations of the kernel and chemical determinations. Storage tests of rice under various conditions were made to know whether there was any movement of inorganic elements other than chlorine, the translocation of which had been found by the author.

By placing rice of relatively high moisture content at 30°C for 20 days, not only chlorine but potassium in embryo and bran layer moved into endosperm. During a longer storage under high temperature and humidity, the translocation of potassium continued even after almost all of chlorine had transferred into endosperm, and small amounts of phosphorus and magnesium also seemed to move inwards.

The total amount of potassium translocated throughout the period of the experiment was far more than that of chlorine, and it was concluded that the movement of chlorine was one of the general phenomenon of inner translocation of electrolytes or water-soluble substances conveyed by the so-called "free" water in the rice kernel.

Determination of Hydrated Water in Soaps by Measurement of Dielectric Constant, Infrared Spectra and X-Ray Diffraction. (Physico Chemical Studies on Higher-Acylcarboxylate-Water Systems. Part III.) (p. *694~698*)

By Syun NOGUCHI

(*Mitsuwa Chemical Laboratory, Marumiya Co. Ltd., Sumida-ku, Tokyo, Japan*)

In the previous work, it was confirmed by the measurement of the dielectric constant that hydrated water is always contained in sodium saturated higher fatty carboxylates and in sodium *trans*-unsaturated one-elaidate, and no in *cis*-unsaturated carboxylate.

Further confirmation was performed for the studies on the infrared spectra and the X-ray diffraction of the sodium soaps mentioned in part II of this series.*

Furthermore, it was again repeated by the measurement of the dielectric constant that hydrated water may still be contained in sodium stearate and laurate even after milling on the rolls, but on the contrary always negative in the case of potassium and lithium soaps.

The results are now described and discussed.

* Part I. *Bull. Agr. Chem. Soc. Japan*, **34**, 416 (1960).
Part II. *ibid.*, **34**, 420 (1960).

Studies on the Requirements of Amino Acids and Vitamins for the Growth of Yeast. Part IX. Mechanism of the Synthesis of β-Alanine by Yeasts.

(p. *699~704*)

By Masahiro TAKAHASHI

(*Food Research Laboratry, Ajinomoto Co., Inc.*)

1. The effect of aspartic acid on the growth of *Sacch. saké* and *Pichia membranaefaciens* was studied. The growth of *Sacch. saké* was slightly inhibited by the addition of aspartic acid to the basal medium, while the growth of *Pichia.* was stimulated by the addition.

2. Cells of *Sacch. saké* contain pantothenic acid, but not β-alanine. On the contrary cells of *Pichia* contain considerable amount of β-alanine besides pantothenic acid.

3. Production of β-alanine by these strains were examined using various substrates. Each substrate was added in concentration of 10^{-5}M of aspartic acid, succinic acid, fumaric acid, malic acid, pyruvic acid, 3 phosphoglyceric acid, glycerophosphate, oxalic acid, propionic acid, malonic acid, β-hydroxypropionic acid or β-alanine. β-alanine content in cells of *Sacch. saké* was increased when propionic acid was used as substrate. Mean while, the content of β-alanine of cells of *Pichia* strain was increased when incubated with aspartic acid.

4. Since pantothenic acid is to be synthesized from β-alanine and pantoic acid, moreover pantothenic acid is found in both strains, it can be concluded that the yeasts are capable of synthesizing β-alanine.

However the no accumulation of β-alanine in *Sacch. saké* may be due to rapid consumption of the substance for pantothenic acid synthesis.

The pathway of β-alanine synthesis seems to be different between these two strains.

Studies on Honey and Pollen. Part III. On the Sugar Composition of Pollen of *Typha lalifolia Linne*

(p. *704~708*)

By Toshiyuki WATANABE, Yoshie MOTOMURA and Kiyoshi ASO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

On the analysis of the pollen of *Typha lalifolia Linne*, 16.0% of moisture, 18.9% of protein, 17.8% of carbohydrate, 1.2% of fat and 3.7% of ash were obtained.

By the methods of paper chromatography, paper ionophoresis and carbon-Celite column chromatography of sugars in the pollen, eighteen spots corresponding to rhamnose, xylose, arabinose, glucose, fructose, kojibiose, nigerose, maltose, isomaltose, sucrose, turanose, leucrose, maltot- riose, raffinose and four higher oligosaccharides were detected and determined.

The main sugars in the pollen were glucose and fructose. The content of monosaccharides including xylose, arabinose and rhamnose were 97% of total sugar.

Glucose, fructose, kojibiose, nigerose, maltose, isomaltose, sucrose, turanose, leucrose and raffinose were detected as same as in honey. Rhamnose, xylose and arabinose were detected in pollen while honey did not contain these sugars.

The varieties and quantities of oligosaccharides in the pollen were less than those of honey, and also oligosaccharides containing ketose were less than those of honey.

New Species and Varieties *Cryptococcoideae* from Grape Musts.

(p. *709~711*)

By Yuwao OHARA, Hideo NONOMURA and Hideo YUNOME

(*Research Institute of Fermentation, Yamanashi University and Research Laboratory, Kotobukiya Co., Ltd.*)

A taxonomic study was made on the three strains of yeasts isolated from grape musts. One of them was recognized as a new species, *Candida vinaria* sp. nov. and other two as new varieties,*Candida fimetaria* var. *diversa* var. nov. and *Torulopses bacillaris* var. *obesa* var. nov. respectively. *C. vinaria* resemcles *C. rugosa* especially in its physiological properties but differs from the latter species in some characteristics; rare formation of pseudomycelium, poor growth in malt extract, absent of pellicle, smaller and shorter cells. *C. fimetaria* var. *diversa* is distinguished from the species by poor development of

"Mycocandida" type pseudomycelium and multiplicity of cell size. *T. bacillaris* var. *obesa* differs from the species in the appearence of the streak culture and the shape of the cells.

New Devices for Feeding Experimental Rats. Part 1. Hanging Feeder. (p. *711～712*)

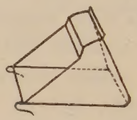
By Michio YAMAGUCHI and Makoto KANDATSU

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Hanging Feeder devised is shown in the figure below. With the hooks attached to the top this feeder is hung at the inside of a cage and food is placed in it. A rat can easily go up to the upper foothold at the front side and eat the food sitting there without scattering it.

This feeder has proven quite satisfactory for dry diet of smaller particle or powder with less than 0.3 per cent loss to the amount eaten, and moreover fully available for wet diet with about 0.5 per cent loss and even for liquid one. And all these diets are kept clean without being contaminated with urine and feces.

The above advantages made us possible to use this feeder in a metabolism cage with full satisfaction. The combined form of three or four of this feeder is most suitable for usual feeding.



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